ENDOCRINOLOGY

PUBLISHED FOR

THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

VOLUME 28 FEBRUARY, 1941 NUMBER 2

THE EFFECT OF THYROXIN AND THYROTROPIC HORMONE ON THE BASAL METABOLISM AND THYROID TISSUE RESPIRATION OF RATS AT VARIOUS AGES[1]

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MANY STUDIES in senescence have centered about the endocrines. The thyroid and pituitary relationship has proved to be of interest, because many of the characteristics of old age resemble those of hypothyroidism (1). In the human being past the age of one year, the basal metabolism declines at a high rate until maturity, at which time the decline becomes retarded and continues at the slower rate for the remainder of life (2). The rat shows a similar trend in its basal heat production (3–8). The respiration of normal mammalian tissues also exhibits a decrease with advancing age (9–12). After maturity the histology of the thyroid reveals a decrease in the gland's function (13, 14) although Dogliotti and Nuti (15) believe that there is a reactivation of the thyroid gland in old age.

The response to thyroxin or desiccated thyroid has been shown to be influenced by the factor of age (9, 16, 17); that is, toxicity of thyroid preparations increases with age. It was the purpose of this research to compare qualitatively and quantitatively the response of the ageing rat to thyroxin and the thyrotropic hormone.[3]

Ingestion of desiccated thyroid or iodine is known to depress the respiration of the thyroid (18, 19).

PROCEDURE

All the rats used were of the Wistar strain, and were grouped according to age and sex. Animals 2, 4, 6, 8, 10 and 12 months of age of both sexes were used, and one group of 18-month-old females. Thyrotoxicosis was induced by the subcutaneous injection of thyroxin (Squibb) solution in the amount of 0.2 mg. per 100 gm. of body weight daily for a period of 6 successive days. The crystalline thyroxin was dissolved in approximately N/100 NaOH. The thyrotropic principle of the anterior pituitary was injected subcutaneously into other rats in the amount of 20 guinea pig units per

Received for publication August 1, 1940.

[1] This investigation was supported by a grant of the National Research Council's Committee on Endocrinology.

[2] The data in this paper are taken from a thesis presented in partial fulfillment of the requirements for the doctorate of philosophy, University of Rochester, June, 1940.

[3] The thyrotropic principle, as antuitrin-T, was supplied through the courtesy of Dr. O. Kamm of Parke, Davis & Company.

100 gm. of body weight daily for a period of 6 successive days. The animals were weighed daily and the dose of each hormone was adjusted accordingly. On the sixth and last day of injection, the basal metabolism of the rat in the post-absorptive condition was determined. The apparatus used for such determinations employed the Haldane principle, moisture, carbon dioxide production and oxygen consumption being determined gravimetrically. This particular form of apparatus was constructed similarly to the one employed by Forbes, Kriss and Miller (20) with a few minor

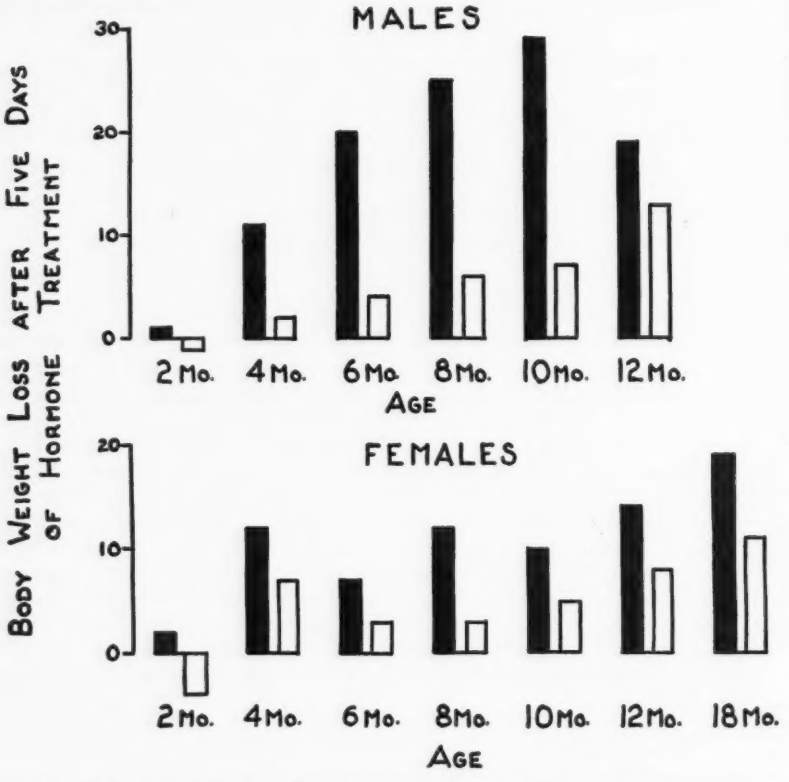

FIG. 1. INFLUENCE OF THYROXIN AND THYROTROPIN ON BODY WEIGHT LOSSES OF MALE AND FEMALE RATS AT DIFFERENT AGES. Block below the line indicates gain in weight. Solid bars, thyroxin treated; white bars, thyrotropic hormone treated.

modifications by Black (6). All measurements were made with the temperature of the respiration chamber between 28°C. and 29°C. The length of experimental period was 6 hours. Basal heat production values are expressed as calories per square meter of body surface per hour, according to the formula of Diack (21).

On the seventh day, the animal, still in the post-absorptive state, was killed by a blow on the head, and the tissues whose respirations were to be studied were removed as quickly as possible and sliced. The oxygen uptake of thyroid tissue was determined by the use of the Warburg constant volume manometers, utilizing the respiration flask of Marsh (22). The medium in which the tissues were placed was a Ringer-

phosphate-glucose solution; the gas space in the flask contained air. Since the thyroid of the rat is small, only single determinations of oxygen consumption were possible.

RESULTS

Body weight changes after hormone administration. The average absolute daily weight changes of each group are shown in figure 1. It is obvious that the weight loss after both hormones became greater with increase of age, but not at equivalent rates for the two sexes. Expressed as percentage of original body weight, the loss after thyroxin actually remained fairly constant beyond the age of 4 months, but after thyrotropin, both relative and absolute losses became steadily greater with age.

Basal metabolism. The values of the indirect heat production of control, thyroxin treated and thyrotropic hormone treated rats are shown in tables 1 and 2 and figure 2. The average basal heat production of the youngest normal males was 44.7 calories

TABLE 1. SUMMARY OF MALE RAT BASAL METABOLISMS

	Sex	Age, mo.	No. in group	Cals./hr.	% change	Cal./sq.m./hr.	% change	R.Q.
Controls	M	2	5	0.995	—	44.7	—	0.73
Thyroxin treated	M	2	5	1.462	+47	68.5	+53	0.73
Thyrotropin treated	M	2	6	1.093	+10	47.7	+ 7	0.73
Controls	M	4	11	1.172	—	42.2	—	0.73
Thyroxin treated	M	4	6	1.933	+65	75.2	+78	0.73
Thyrotropin treated	M	4	6	1.315	+11	51.3	+22	0.73
Controls	M	6	6	1.099	—	35.2	—	0.74
Thyroxin treated	M	6	6	1.832	+67	57.5	+63	0.74
Thyrotropin treated	M	6	6	1.548	+41	47.9	+36	0.72
Controls	M	8	6	1.211	—	35.7	—	0.73
Thyroxin treated	M	8	6	2.148	+77	61.6	+73	0.73
Thyrotropin treated	M	8	6	1.639	+35	52.8	+48	0.73
Controls	M	10	6	1.456	—	37.5	—	0.73
Thyroxin treated	M	10	6	2.377	+63	63.8	+70	0.73
Thyrotropin treated	M	10	6	1.877	+30	52.0	+39	0.73
Controls	M	12	6	1.383	—	37.4	—	0.73
Thyroxin treated	M	12	6	2.426	+75	64.5	+75	0.73
Thyrotropin treated	M	12	6	1.571	+14	45.8	+23	0.73

per square meter per hour. The heat production declined rapidly to a value of 35.5 calories per square meter per hour in the 6-month-old group. From here on it remained relatively constant although there was a tendency for it to increase in the older groups. We believe this decline correctly represents the course of the metabolism the first 6 months, and it confirms the findings of Black and Murlin (23) for total metabolism in the rat. The number of determinations, however, does not permit us to make this assertion with too much assurance. With regard to the control females, it can be said that in the younger groups the metabolism was below that found amongst the males, but not between the ages of 6 and 12 months. The metabolism declined with age until 6 months and then remained fairly constant, although the heat production of the 18-month-old female rats was considerably lower than at 12 months.

The resting metabolism of thyroxin treated males, 2 months of age, was 53% above that of the controls; at 4 months this hypermetabolism rose to +78% and

remained approximately at the same high level as far as these studies have gone. Thyrotropic hormone had little effect on the 2-month-old male rats, but thereafter the metabolism with this hormone rose to a value of +48% at 8 months, and then decreased to +23% with the oldest males studied.

The increased heat production of females after thyroxin administration became greater with age up to the time they were 6 months old. The hypermetabolism then dipped to a new low point of +37% at 8 months and again climbed gradually to a new high point of +71% for the 18-month-old females. Likewise the thyrotropic hormone had very little effect on the 2-month-old females, but the response after

TABLE 2. SUMMARY OF FEMALE RAT BASAL METABOLISMS

	Sex	Age, mo.	No. in group	Cal./hr.	% change	Cal./sq.m./hr.	% change	R.Q.
Controls	F	2	7	0.751	—	40.9	—	0.73
Thyroxin treated	F	2	6	0.971	+29	51.8	+27	0.73
Thyrotropin treated	F	2	7	0.842	+12	43.9	+ 7	0.73
Controls	F	4	6	0.850	—	38.6	—	0.73
Thyroxin treated	F	4	5	1.226	+44	55.5	+44	0.73
Thyrotropin treated	F	4	6	1.138	+34	49.8	+29	0.73
Controls	F	6	6	0.894	—	36.9	—	0.73
Thyroxin treated	F	6	6	1.350	+51	58.7	+59	0.73
Thyrotropin treated	F	6	6	1.166	+30	50.7	+37	0.73
Controls	F	8	6	0.966	—	37.6	—	0.73
Thyroxin treated	F	8	6	1.355	+40	51.5	+37	0.74
Thyrotropin treated	F	8	6	1.176	+22	46.2	+23	0.73
Controls	F	10	6	0.943	—	37.0	—	0.73
Thyroxin treated	F	10	6	1.367	+44	54.7	+48	0.73
Thyrotropin treated	F	10	6	1.286	+36	48.9	+32	0.73
Controls	F	12	5	1.040	—	38.4	—	0.73
Thyroxin treated	F	12	6	1.563	+50	56.2	+49	0.73
Thyrotropin treated	F	12	5	1.343	+29	46.3	+20	0.73
Controls	F	18	6	1.001	—	35.4	—	0.73
Thyroxin treated	F	18	6	1.652	+65	60.4	+71	0.73
Thyrotropin treated	F	18	6	1.194	+19	42.7	+21	0.73

this age increased to 37% at 6 months, and thereafter declined as it did amongst the older male rats.

It is evident from the graphic representation of the data (fig. 2) that metabolism responded more abruptly in the young males than in the young females to thyroxin, but not to thyrotropic hormone. Beyond 6 months the stimulating effect of thyroxin progresses at about the same rate in the two sexes, but the levels attained are lower in females, as would be expected. The same can be said of thyrotropic hormone.

Two explanatory statements are in order at this point, the first, that no attempt was made to find equivalent doses of the two hormones. This would scarcely be desirable until a pure preparation of the thyrotropic hormone is available. The second is that the preparation used (Parke, Davis, antuitrin-T) is known to contain gonadotropic factors, and the sex difference in response, both absolute and relative, may be due in part to the difference in concentration of male and female factors contained.

Thyroid tissue respiration. The oxygen consumption of thyroids removed from control normal rats, as can be seen from figure 3, was at a peak when the animal was

4 months of age. This was evident in both sexes. From this point on, there was a rapid decline in thyroid respiration with the advance of age. Thyroid tissue of females respired regularly at a higher rate than did the thyroid of male rats.

The decrease of thyroid metabolism brought about by thyroxin injections was quite large. The depression in both males and females ranged from 20% to 50%

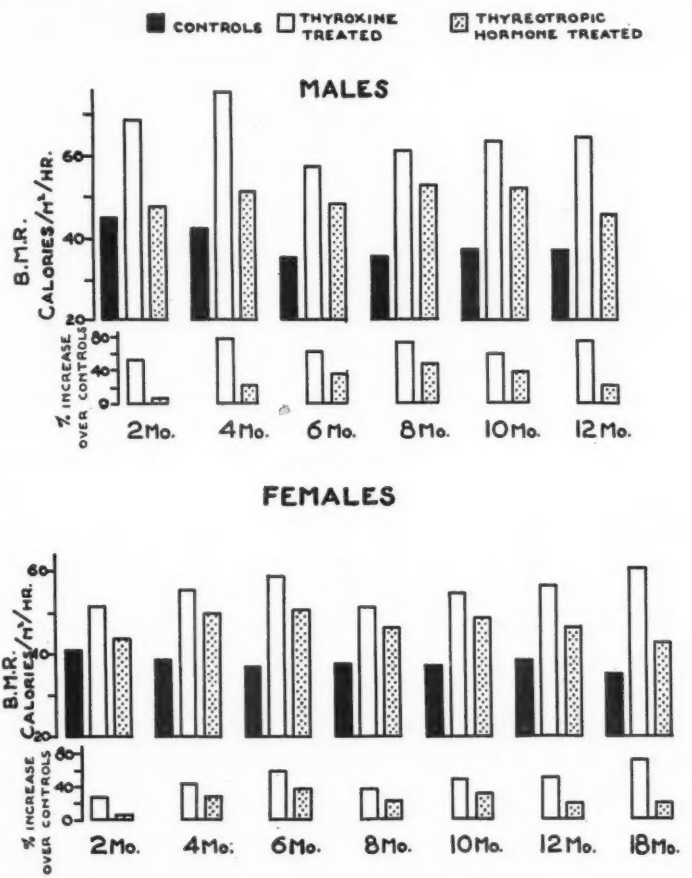

Fig. 2. Basal metabolism of male and female rats at different ages and the effects thereon of uniform doses of thyroxin and thyrotropic hormone.

below the normal values. There was, however, no definite correlation between the age of the animal and the extent of the depression produced.

The thyrotropic hormone, however, as would be expected from the known histological effects, produced increased thyroid respiration in every age group but one. The least response was at the age of 4 months in both sexes; in fact, the response here can be considered negligible. From this group on the response became greater with age when expressed as percentage increase over controls, but the correlation with age is not high.

It is of interest to note here that the thyroids of animals treated with either thyroxin or thyrotropic hormone increased in dry weight. Histological examination of the glands revealed that these animals treated with thyroxin possessed thyroids with accumulating colloid, while those animals treated with thyrotropin exhibited the typical active thyroid hyperplasia.

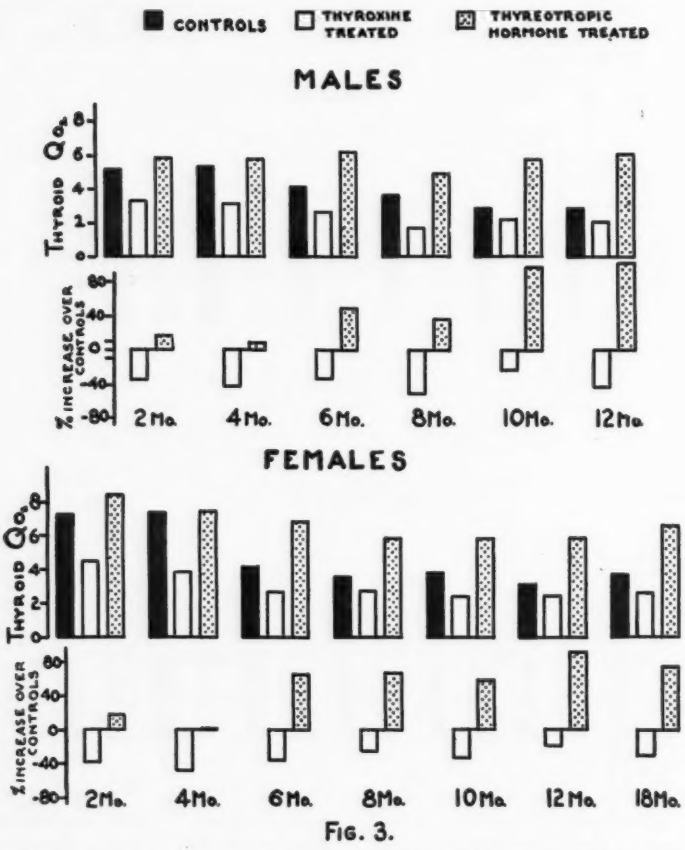

FIG. 3.

Fig. 3. THE RESPIRATORY METABOLISM OF THYROID GLAND TISSUE IN MALE AND FEMALE RATS OF DIFFERENT AGES AND THE EFFECTS THEREON OF UNIFORM DOSES OF THYROXIN AND THYROTROPIC HORMONE.

DISCUSSION

The body weight changes and the resting metabolism of the entire organism clearly indicate that the toxicity, if such it may be called, of thyroxin and thyrotropic hormone does increase with age. The factor of age is also clearly evident in the response elicited by the thyrotropic hormone in the respiration rate of the thyroid gland, the organ which is most directly affected by the administration of this pituitary hormone. The hypermetabolism of the intact organism gives indications of declining in the very old animals. If we are to accept the idea that increased thyroid respiration produced by thyrotropic stimulation is an index of increased thyroid hormone produc-

tion and secretion into the circulatory system, then to explain the differences in response between the intact organism and the thyroid gland, we must postulate one of two theories. First, there is an accumulation with age of anti-thyroid substance. Or, secondly, the colloid of the older animal has a low content of active thyroid hormone —the mechanism itself suffers from senescence.

If perchance there is an accumulation of an antagonistic substance in the body of the old rat, it is definitely anti-thyroid and not anti-thyrotropic, as might be expected. If an anti-thyrotropic substance were the limiting factor, the thyroid gland would have indicated its presence by a lessened respiratory response to the injected thyrotropic hormone in the old animals; but it did not. Instead, the thyroid metabolism under this stimulus rose to higher levels with the increase of age. The point of antagonism lies between the thyroid and the remainder of the body and therefore must be considered as an anti-thyroid effect.

The second possibility may prove to be an actuality, but as yet no one has assayed the thyroids of animals of various ages for their active thyroid hormone content. This will surely have to be done in order to clarify our views.

The findings of Turner and Cupps (24) on the thyrotropic hormone content of the pituitary of rats throughout life agree fairly well with the histological study of Cooper (13) and with the results of this investigation. Cooper observed from her histological analysis that the secretory and absorptive activity of the thyroid before puberty is marked, although the gland always maintains a small reserve of colloid in its small vesicles. At puberty, however, the thyroid metabolic activity is at a maximum, and since the thyroid is unable to manufacture the hormone as rapidly as it is secreted, the vesicles contain very little colloid. Turner and Cupps found that the highest concentration of the thyrotropic hormone in the pituitary occurs at puberty in the rats. The present investigation has demonstrated that the respiration of thyroids of rats at puberty, cubic millimeter per milligram, is at a maximum. Coincident with this high thyroid Q_{O_2}, the weight of the thyroid is at a minimum. In the light of this coincidence, it is understandable why thyrotropic hormone has little if any effect on thyroid metabolism at this period in the rat's life. The thyroid respiration is at a maximum and further stimulation apparently is impossible.

The results presented may have a clinical bearing from the point of view that thyroxin will depress thyroid respiration, even though it elevates the respiration of other tissues and the metabolism of the intact organism. This depression of thyroid respiration is accompanied by a storage of colloid in the follicles of the thyroid with consequent flattening of the acinar epithelium. It is quite possible that the thyroid gland of individuals taking thyroid over a long period of time may lose its characteristic ability to return to the active state after cessation of medication, and thus compel the recipient to continue medication in larger amounts. Would it not be sound physiology to give those patients needing thyroid treatment only temporarily an injection of thyrotropic hormone intermittently along with the ingestion of desiccated thyroid for the purpose of maintaining the thyroid gland in a normal condition, so that the thyroid may be able to resume function immediately upon the cessation of such medication?

SUMMARY

Body weight losses during the administration of either thyroxin or thyrotropic hormone is influenced by the age of the animal. That is to say, the younger the animal, the more resistant it is to body weight changes. In spite of this body weight loss, the weight of the thyroid gland increased upon thyrotropic hormone treatment.

The basal metabolism of the normal rats studied declined rapidly during the first

few months of life, and then reached a rather constant level. The hypermetabolism produced by either thyroxin or thyrotropic hormone in the young animal was less than that produced in the older animal.

Normal thyroid tissue respiration declined rapidly after the rat was 4 months old. Thyroxin depressed the respiration of thyroid tissues while the thyrotropic hormone produced gradually increasing oxygen consumption of the gland in the ageing rat. The clinical implications are discussed.

REFERENCES

1. Carlson, A. J.: Problems of ageing, Chapter XIII. Williams & Wilkins, Baltimore, 1939.
2. Boothby, W. M., J. Berkson and H. L. Dunn: *Am. J. Physiol.* 116: 468. 1936.
3. Davis, J. E., and A. B. Hastings: *Am. J. Physiol.* 109: 683. 1934.
4. Davis, J. E.: *Am. J. Physiol.* 119: 28. 1937.
5. Sherwood, T. C.: *J. Nutrition* 12: 233. 1936.
6. Black, A.: *J. Nutrition* 17: 361. 1939.
7. Ashworth, U. S., S. Brody and A. G. Hogan: *Mo. Agr. Exper. Sta., Research Bull.* 166: 71. 1932
8. Horst, K., L. B. Mendel and F. G. Benedict: *J. Nutrition* 7: 277. 1934.
9. McEachern, D.: *Bull. Johns Hopkins Hosp.* 50: 287. 1932.
10. Victor, J., and J. S. Potter: *Britt. J. Exper. Path.* 16: 243. 1935.
11. Pearce, J. M.: *Am. J. Physiol.* 114: 255. 1936.
12. Adams, P. D.: *Arch. Dermat. Syphilol.* 36: 606. 1937.
13. Cooper, E. R. A.: The histology of the more important human endocrine organs at various ages, 1925. Oxford Med. Publication.
14. Loeb, L., and R. M. Simpson: *Science* 88: 433. 1938.
15. Dogliotti, G. C., and G. N. Nuti: *Endocrinology* 19: 289. 1935.
16. Cameron, A. T., and J. Carmichael: *J. Biol. Chem.* 45: 69. 1920–21.
17. Bodansky, M., and V. B. Duff: *Endocrinology* 20: 541. 1936.
18. Cohen, R. A.: *Proc. Soc. Exper. Biol. & Med.* 32: 1446. 1935.
19. Sturm, A.: *Ztschr. f. d. ges. exper. Med.* 74: 555. 1930.
20. Forbes, E. B., M. Kriss and R. C. Miller: *J. Nutrition* 8: 535. 1934.
21. Diack, S. L.: *J. Nutrition* 3: 289. 1930.
22. Marsh, M. E.: *J. Nutrition* 13: 109. 1937.
23. Black, A., and J. R. Murlin: *J. Nutrition* 17: 347. 1939.
24. Turner, C. W., and P. T. Cupps: *Endocrinology* 24: 650. 1939.

THE EFFECT OF THYROXIN AND THYROTROPIC HORMONE ON LIVER AND KIDNEY TISSUE RESPIRATION OF RATS OF VARIOUS AGES[1]

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IN THE PRECEDING PAPER (1) the effect of age on the metabolic response of the entire organism and of thyroid tissue to treatment of rats with these hormones was discussed. Carried on simultaneously with the other, the present investigation sought to elucidate effects on the respiratory activity of liver and kidney tissues in the same animals.

PROCEDURE

The rats were grouped as to age and sex, and the ages at which the animals were studied in respect to these tissue respirations were the same as in the previous paper; namely, 2, 4, 6, 8, 10 and 12 months, and 18 months for one group of females. Crystalline thyroxin (Squibb) dissolved in N/100 NaOH was injected subcutaneously into the test rats in the amount of 0.2 mg. per 100 gm. of body weight per day for 6 successive days. Thyrotropic hormone[3] was injected into other animals in the amount of 20 guinea pig units per 100 gm. of body weight per day for 6 successive days also. On the seventh day, the animals in a post-absorptive condition were killed, and the tissues whose respirations were to be determined were removed and sliced as rapidly as possible. The kindey cortex and liver slices were pooled in separate Petri dishes, containing a Ringer phosphate glucose solution. Uniform representative slices were then placed in the Marsh respiration flask (2) also containing the Ringer phosphate glucose solution as a medium. The gas space of these flasks when attached to constant volume manometers of the Warburg type, contained air. Within 30 minutes from the time the rat was killed, the determination of tissue respiration was under way. Both kidney cortex and liver tissues were run in triplicate, and they checked with one another remarkably well. The length of period for oxygen consumption was 90 minutes. By the use of this type of respiration flask, it was possible to determine the Q_{O_2}, Q_{CO_2} and consequently the respiratory quotient in the same flask, although appropriate controls were necessary.

RESULTS

This investigation can be divided into two separate projects. The first, which was begun in the fall of 1937, consisted only in the study of hepatic and renal tissue respiration of control and thyroxin treated animals in a postabsorptive condition (without

Received for publication August 1, 1940

[1] This investigation was supported by a grant of the National Research Council's Committee on Endocrinology.

[2] The data presented are part of a thesis submitted in partial fulfillment of the requirements for the degree of doctor of philosophy.

[3] The thyrotropic factor of the anterior pituitary was graciously supplied by Dr. Oliver Kamm of Parke, Davis & Co.

food for 18 to 24 hours). The second portion of this research was begun in the fall of 1938 and included a study of the effects of thyrotropic hormone as well as of thyroxin, both on the basal metabolism and on the respiration of kidney, liver and thyroid tissue. The results from basal metabolism and thyroid respiration studies, reported elsewhere (1), belong to this second project. Since the basal metabolism was deter-

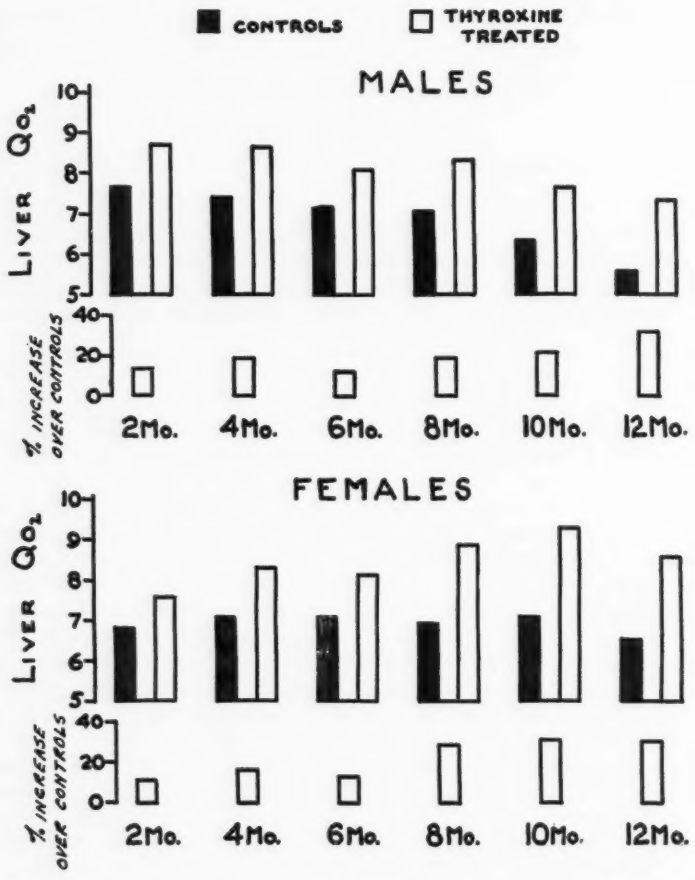

Fig. 1. THE RESPIRATORY METABOLISM OF LIVER TISSUE OF MALE AND FEMALE RATS AT DIFFERENT AGES, ALL FASTED 18 TO 24 HR. The effect of a uniform dose of thyroxin is shown.

mined on the sixth day of hormone administration on animals in the post-absorptive condition, and since tissue respirations were determined on the seventh day with the animals still in the post-absorptive state, the treatment of rats here differed from that of animals in the first project in that they were without food 24 hours longer. This time difference affected the results, thus necessitating a separation of data.

The results of the first project are shown in figures 1 and 2. Amongst the male rats, the Q_{O_2} and Q_{CO_2} of liver tissue from both control and thyrotoxic animals decreased with increase of age. The slopes of these declines, however, differ in such a

manner as to bring out the increased response of the older animal to thyroxin. With increase of age, the percentage increase of oxygen consumption and of carbon dioxide production rose steadily from approximately 10% to 30%.

In the hepatic tissue of female rats from 4 to 12 months of age, the Q_{O_2} and Q_{CO_2} of normal liver showed only a slight downward trend with age; but accompanying

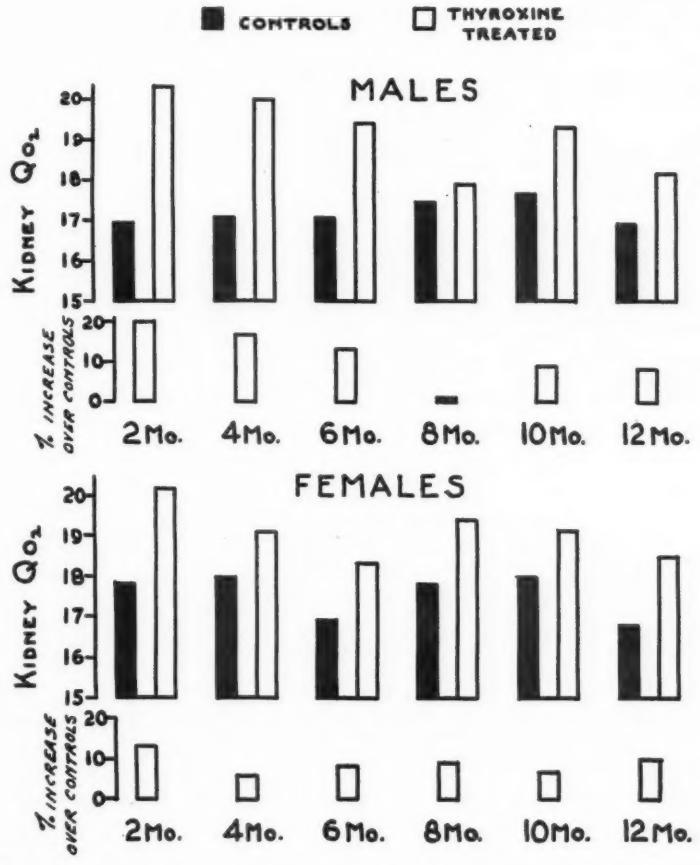

Fig. 2. The respiratory metabolism of kidney tissue of male and female rats at different ages, all fasted 18 to 24 hr. The effect of a uniform dose of thyroxin is shown.

this downward trend, the response to thyroxin became even greater with advancing age than in males.

The respiration of kidney tissue as the result of the particular dose of thyroxin given was elevated about 10% on the average. However, there was no definite correlation between age and the respiration of renal tissue from either controls or thyrotoxic animals. Kidney tissue did not respond to thyroxin as did liver tissue; this has been noticed by others (3–6).

The respiratory quotients of renal and hepatic tissue (not shown) remained un-

TABLE 1. SUMMARY OF MALE RAT TISSUE RESPIRATIONS

	Age mo.	No. in group	Liver					Kidney				
			Q_{O_2}	% change	Q_{CO_2}	% change	R.Q.	Q_{O_2}	% change	Q_{CO_2}	% change	R.Q.
Controls	2	5	7.53	—	3.81	—	0.50	17.4	—	14.0	—	0.80
Thyroxin treated	2	5	9.07	+20	4.74	+24	0.52	19.8	+14	16.3	+16	0.82
Thyrotropin treated	2	6	7.83	+ 4	4.19	+10	0.53	17.3	− 1	13.9	− 1	0.80
Controls	4	6	7.07	—	3.94	—	0.56	17.2	—	13.9	—	0.81
Thyroxin treated	4	6	8.69	+23	4.38	+11	0.51	19.1	+17	15.4	+11	0.81
Thyrotropin treated	4	6	7.49	+ 6	3.82	− 4	0.51	18.0	+ 4	15.1	+ 9	0.84
Controls	6	6	6.45	—	3.32	—	0.52	16.4	—	12.9	—	0.79
Thyroxin treated	6	6	8.45	+31	4.38	+32	0.52	18.6	+13	14.8	+15	0.80
Thyrotropin treated	6	6	7.62	+18	4.00	+21	0.53	16.3	− 1	12.7	− 2	0.78
Controls	8	6	6.75	—	3.41	—	0.50	16.6	—	13.3	—	0.80
Thyroxin treated	8	6	8.94	+34	4.40	+29	0.50	18.5	+11	14.9	+12	0.81
Thyrotropin treated	8	6	8.13	+20	3.99	+17	0.49	17.2	+ 4	13.2	− 1	0.77
Control	10	6	6.69	—	3.46	—	0.52	15.3	—	12.2	—	0.80
Thyroxin treated	10	6	8.98	+34	4.58	+32	0.50	18.5	+21	15.2	+25	0.80
Thyrotropin treated	10	6	7.73	+16	3.78	+ 9	0.49	16.9	+11	13.6	+11	0.82
Controls	12	6	6.39	—	3.01	—	0.47	16.3	—	12.6	—	0.78
Thyroxin treated	12	6	8.58	+34	4.09	+36	0.48	17.8	+ 9	14.4	+14	0.81
Thyrotropin treated	12	6	7.79	+22	3.78	+26	0.49	17.8	+ 9	14.0	+11	0.78

TABLE 2. SUMMARY OF FEMALE RAT TISSUE RESPIRATIONS

	Age, mo.	No. in group	Liver					Kidney				
			Q_{O_2}	% change	Q_{CO_2}	% change	R.Q.	Q_{O_2}	% change	Q_{CO_2}	% change	R.Q.
Control	2	7	8.41	—	4.26	—	0.51	18.4	—	15.2	—	0.82
Thyroxin treated	2	6	9.66	+15	4.90	+15	0.51	20.2	+10	17.2	+13	0.85
Thyrotropin treated	2	7	8.21	− 2	4.26	0	0.52	18.5	+ 1	14.8	− 3	8.80
Controls	4	6	7.15	—	3.99	—	0.56	18.1	—	14.9	—	0.82
Thyroxin treated	4	6	8.45	+18	4.14	+ 4	0.49	18.7	+ 3	15.0	+ 1	0.81
Thyrotropin treated	4	6	7.54	+ 6	3.66	− 8	0.49	18.6	+ 3	15.5	+ 4	0.83
Controls	6	6	7.05	—	3.65	—	0.52	17.3	—	14.5	—	0.84
Thyroxin treated	6	6	8.46	+20	4.12	+13	0.49	19.0	+10	15.8	+10	0.83
Thyrotropin treated	6	6	7.52	+ 7	3.72	+ 1	0.49	18.1	+ 5	14.7	+ 5	0.81
Controls	8	6	6.86	—	3.41	—	0.50	17.5	—	14.1	—	0.82
Thyroxin treated	8	6	8.71	+27	4.11	+21	0.47	20.1	+15	16.6	+18	0.85
Thyrotropin treated	8	6	7.85	+14	3.68	+ 8	0.47	18.9	+ 8	15.2	+ 8	0.79
Controls	10	6	6.94	—	3.23	—	0.47	17.2	—	13.8	—	0.81
Thyroxin treated	10	6	9.09	+31	4.24	+31	0.47	20.5	+19	16.7	+21	0.82
Thyrotropin treated	10	6	7.83	+13	3.56	+10	0.46	18.9	+10	15.4	+12	0.82
Controls	12	5	6.58	—	3.46	—	0.53	16.7	—	13.7	—	0.82
Thyroxin treated	12	6	8.42	+28	4.21	+22	0.50	20.8	+25	17.6	+28	0.85
Thyrotropin treated	12	5	7.87	+20	3.80	+10	0.48	18.1	+ 8	14.4	+ 5	0.79
Controls	18	7	7.01	—	3.35	—	0.47	17.0	—	13.7	—	0.81
Thyroxin treated	18	6	9.26	+32	4.49	+34	0.48	20.6	+21	16.8	+23	0.82
Thyrotropin treated	18	6	7.69	+10	3.67	+10	0.48	17.1	+ 1	13.5	− 1	0.79

altered by thyrotoxicosis. It varied from 0.53 to 0.61 in liver and from 0.87 to 0.93 in kidney—the usual range in these organs of the rat fasted 18 to 24 hours. Thus it is apparent that experimental hyperthyroidism does not qualitatively change the metabolism of tissues, but only does so quantitatively. This is in confirmation of other investigators (4, 7).

The results of the second project in which the respiration of tissues of animals fasted from 42 to 48 hours was studied are shown in tables 1 and 2 and in figures 3 and 4. In both sexes respiration of normal hepatic tissue declined rapidly during the first

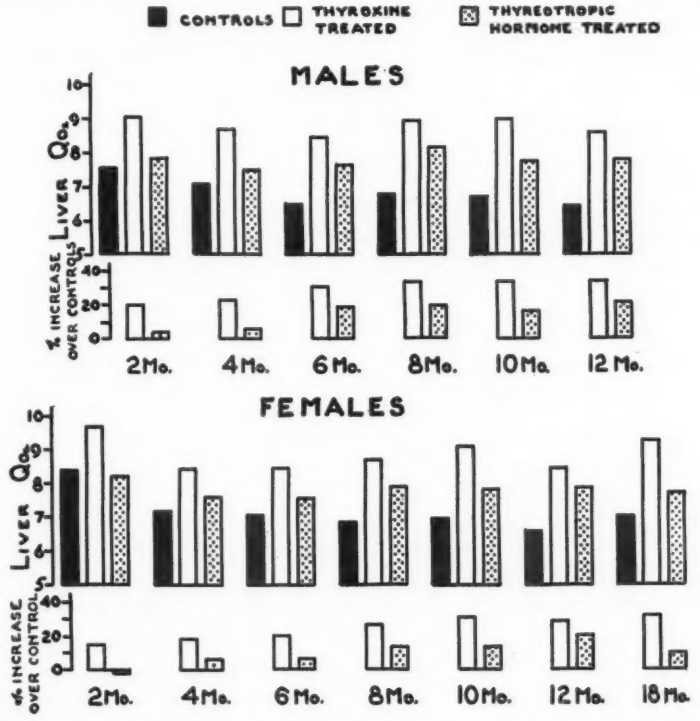

Fig. 3. THE RESPIRATORY METABOLISM OF LIVER TISSUE OF MALE AND FEMALE RATS, AT DIFFERENT AGES, ALL FASTED 48 HR. BEFORE KILLING. The effects of uniform doses of thyroxin and thyrotropic hormone are shown.

4 months of life, and thereafter was fairly constant. The low point fell at 12 months for both. The same was true of normal kidney tissue except that the early decline was somewhat less abrupt. The low point fell at 10 months for males and 12 for females. The response of liver to thyroxin became greater with age, just as it did in the earlier experiments. The thyrotropic hormone response also became greater with the advancing age of the animal.

The metabolism of kidney tissue of thyroxin treated male rats declined with age. Expressing the response of this tissue as percentage increase over the control values, there was no correlation between the age of the rat and the response elicited from the renal tissue because of a very discrepant result in the 10-month group. It is readily seen from figure 4 that this is explained by the exceptionally low value obtained in

the controls of this age. On the other hand, kidney tissue of female rats, similarly treated, responded to thyroxin administration in a more regular fashion. The response, expressed as percentage increase, became greater with age. In both males and females, elevated kidney respiration, resulting from thyrotropic hormone administration in general increased with advancing age of the animal. Again there is an exceptional group—this time at 6 months for males.

Liver and kidney weights became greater following treatment with either hormone used in these experiments. No correlation existed between the age of the ani-

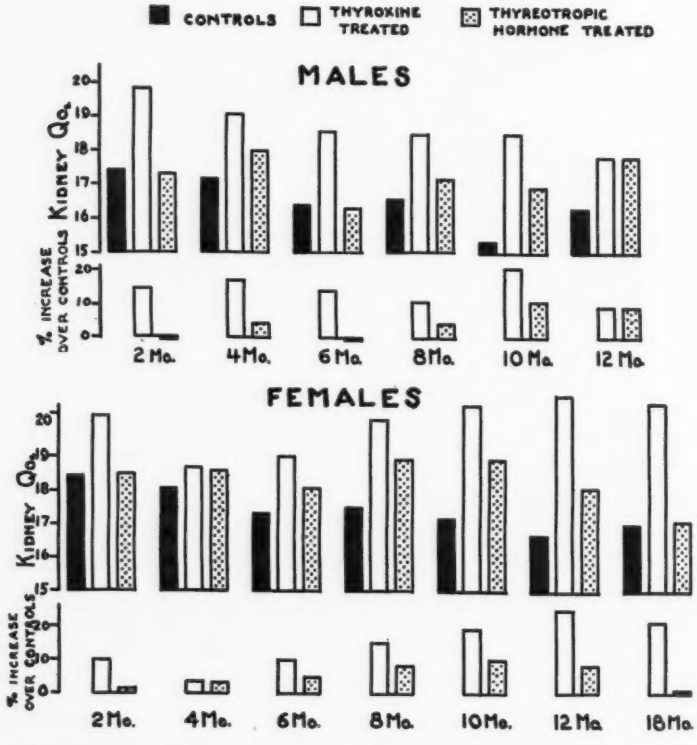

Fig. 4. The respiratory metabolism of kidney tissue of male and female rats at different ages, all fasted 48 hr. The effects of uniform doses of thyroxin and thyrotropic hormone are shown.

mal and the extent of hypertrophy. Fresh liver and kidney weights increased between 10% to 20% as a result of experimental hyperthyroidism.

As a result of the extension of the length of fast, the respiratory quotients of both liver and kidney tissue decreased noticeably. Still neither hormone altered the R.Q.

DISCUSSION

There is no question that age is a factor both in normal tissue respiration and in the response of tissues to thyroxin or thyrotropic hormone administration. However, the response to the thyroid stimulating hormone (thyrotropin) did not closely parallel the response elicited by thyroxin through the various age groups. The possibility

of accumulation of an anti-thyroid substance within the organism or the possible differences between young and old animals in regard to the content of active hormone of the thyroid has been discussed in the previous publication (1).

We are confronted with the problem as to why the liver and kidney tissue respirations of female rats proceed at a higher rate than those of the males, while the basal metabolism of the former is less than that of the latter. It cannot be due to sex differences in liver and kidney size, because, as found in this investigation, the ratios of both liver and kidney weights to total weights are greater in the females than in the males after the age of 2 months. Quite probably the larger proportion of skeletal muscle in males is sufficient to account for their higher basal rates, assuming that the tissue respiration of resting muscle is the same in both sexes. However, it is possible that male muscle tissue respires at the higher rate. So far as we can discover, nobody has compared the two sexes in this respect in any animal.

Hepatic respiratory quotients of the animals in the first series of experiments, fasted 18 to 24 hours, ranged mostly between 0.50 and 0.60. Fasting has been known to reduce the R.Q. of liver tissue (8). There are 4 possible explanations of low respiratory quotients of hepatic tissue, which is glycogen poor after a 24-hour fast. First, it might signify the conversion of fatty acids to carbohydrate; secondly, desaturation of fatty acids; thirdly, the oxidation of fats, which are mobilized in the liver during fasting, is carried only to the ketone body stage; and lastly, the carbon dioxide produced locally is utilized in urea formation. Liver tissue of rats in the second series of experiments, fasted 42 to 48 hours, exhibited still lower respiratory quotients, some of which were as low as 0.40. Respiratory quotients of renal tissue also showed reductions as a result of this prolonged post-absorptive state. Evidently the metabolism of fats is predominantly involved in the liver of an animal, without food for 2 days.

The conversion of fatty acids to carbohydrate in fasting livers was made plausible by the observations of Gemmill and Holmes (9)—not refuted, as some appear to believe, by Cori and Shine (10), and noted by Marsh (11)—and supported by recent observations of Blixenkrone-Møller (12, 13). This author shows that the increased oxygen consumption of the perfused cat liver is not accounted for by β-oxidation and formation of only one molecule of β-oxybutyric acid from each molecule of higher fatty acid, but is more nearly accounted for by oxidative cleavage into 4-C groups without liberation of CO_2. Upon perfusion with Na butyrate (13) it was found that only about 20% of the supplement went to form ketone bodies, and the remainder appeared as carbohydrate, with a D:N ratio higher than 20. It is evident that both these processes (desaturation and conversion of the resulting butyrate to glucose) would account for very low R.Q.'s. Further diminution of glycogen in the liver, more especially by pancreatectomy (13), would reduce the R.Q. still more by the oxidation of butyrate to ketone bodies. Blixenkrone-Møller's work requires confirmation.

An excellent investigation published by Sternheimer (15) gives us some clue as to what happens to the liver chemistry following an injection of thryoxin. Liver glycogen loss was at a maximum 48 hours after the injection, but soon rose to above normal levels. Liver protein reached a peak when liver glycogen values were low. Liver sugar, blood sugar and lactic acid content of liver remained unchanged until glycogen rose to high levels. In his discussion he attempts to explain liver hypertrophy encountered in experimental hyperthyroidism (15, 16, 17) by the analogy of low glycogen content in growing tissues. His results show that liver weight is at a peak when liver glycogen is at a minimum following the thyroxin injection. Histological examination of liver during this period portrayed changes similar to those observed in regenerating liver with increasing number of mitoses.

SUMMARY

The respiration of normal liver and kidney is a function of age; with increase of age, there is a gradual decrease of oxygen consumption and a corresponding decrease of respired carbon dioxide. Age also proved to be a factor in the response of both renal and hepatic tissue metabolism to either thyroxin or the thyrotropic principle of the anterior pituitary. In the very old animals the increased respiration of these tissues elicited by the thyroid stimulating hormone was lessened.

The respiratory quotient of liver and kidney slices is altered by the length of time the animal is without food. The longer the fasting period, the lower is the resulting respiratory quotient. Oxidative desaturation of fatty acids, conversion of the resulting 4-C fatty acids to carbohydrate and oxidation to ketone bodies could account for the very low R.Q.'s found in liver.

Both the liver and kidneys hypertrophied following the administration of either thyroxin or the thyrotropic hormone. The extent of the hypertrophy varied between 10% and 20% and could not be correlated with age of the rat.

REFERENCES

1. Belasco, I. J., and J. R. Murlin: *Endocrinology* 28: 145. 1941.
2. Marsh, M. E.: *J. Nutrition* 13: 109. 1937.
3. McEachern, D.: *Bull. Johns Hopkins Hosp.* 56: 145. 1935.
4. Victor, J., and D. H. Andersen: *Am. J. Physiol.* 120: 154. 1937.
5. Ebina, T.: *Tohoku J. Exper. Med.* 19: 139. 1932.
6. Rohrer, A.: *Biochem. Ztschr.* 145: 154. 1924.
7. Meyer, O. O., C. McTiernan and J. C. Aub: *J. Clin. Invest.* 12: 723. 1933.
8. Elliott, K. A. C., M. E. Greig and M. P. Benoy: *Biochem. J.* 31: 1003. 1937.
9. Gemmill, C. L., and E. G. Holmes: *Biochem. J.* 29: 338. 1935.
10. Cori, C. F., and W. M. Shine: *Science* 82: 134. 1935.
11. Blixenkrone-Møller, N.: *Ztschr. f. physiol. Chem.* 252: 117. 1938.
12. Blixenkrone-Møller, N.: *Ztschr. f. physiol. Chem.* 252: 137. 1938.
13. Lundsgaard, E.: *Bull. Johns Hopkins Hosp.* 63: 90. 1938.
14. Sternheimer, R.: *Endocrinology* 25: 899. 1939.
15. Cameron, A. T., and F. A. Sedziak: *Am. J. Physiol.* 58: 7. 1921.
16. MacKay, E. M., and L. L. MacKay: *J. Nutrition* 4: 33. 1931.
17. Murphy, R., S. Lowther and L. Pagniello: *Am. J. Physiol.* 124: 110. 1938.

MAMMALIAN AND AVIAN ASSAYS OF HYPOPHYSEAL LACTOGENIC PREPARATIONS[1]

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AT THE THIRD CONFERENCE on the Standardization of Hormones, held in Geneva in August 1938 (1), arrangements were made for establishing an international standard of the lactogenic (crop-sac stimulating) hormone of the anterior pituitary. Since then contributions from nine different sources were offered towards such a standard, and these were mixed and converted into 10 mg. tablets. An attempt was made to have each tablet contain 10 Riddle-Bates units per mg. Some laboratories found that the standard contained slightly more and others slightly less (this laboratory and Riddle's found it contained less) than 10 Riddle-Bates units per mg. But the variation was negligible and "to simplify calculation" the international unit was defined as: "the specific activity contained in 0.1 milligramme (= 100 gamma) of the standard preparation" (2). The Conference was unable, on the information before it, to "recognize any test as giving sufficiently quantitative results for the comparative assay of preparations in relation to this standard, except those which depend on the measurement or observation of growth produced in the crop gland of the pigeon or dove."

Since the discovery of the lactogenic hormone by Stricker and Grüter (3) in 1928, several methods of assaying it in mammals have been available. Probably the method most closely resembling the original test of Stricker and Grüter who used rabbits that had ovulated and thus come under the influence of the corpus luteum, was that of Gardner and Turner (4, 5), employing the pseudopregnant rabbit. Corner (6) and others (7, 8) had also shown that just mature ovariectomized rabbits that had never ovulated could be used to test lactogenic activity of pituitary extracts. Nelson and Pfiffner (9) were the first to demonstrate the effect of lactogenic hormone in the normal and ovariectomized guinea pigs; and Nelson (10) also used hysterectomized pregnant guinea pigs in assaying lactogenic extracts. Ovariectomized guinea pigs were used as assay animals in our studies (11) on the purification of lactogenic hormone, and after the introduction of the pigeon test by Riddle and co-workers (12, 13), we continued to use the guinea pig test in parallel with the pigeon assay in order to determine whether crop and mammary stimulation were caused by the same substance. Since lactogenic hormone, shown to be homogeneous in electrophoretic (14) and solubility studies (15), possesses crop and mammary stimulating properties, it should not be necessary to continue using both assay methods. However, some laboratories may find it more convenient to use guinea pigs than doves or pigeons, and, of course, an easily-standardized mammalian form is indispensable in studies on lactation.

The purpose of this communication is, therefore, to describe some of our experiences in an attempt to establish the guinea pig as an acceptable animal for assaying lactogenic hormone and also to show the relationship between the effective dosages of the international standard and other preparations of lactogenic hormone in the guinea pigs and pigeons used in this laboratory.

Received for publication September 25, 1940.

[1] This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

TABLE 1. COMPARISON OF THE PIGEON CROP-SAC RESPONSE TO SUBCUTANEOUS INJECTIONS OF LACTOGENIC HORMONE PREPARATIONS *LP.1* AND *L 250 S*

No. of pigeons	Total dose in mg.	Average weight of crop-sacs, in grams	
		L 250 S	*LP.1*
5	1.0	3.86	2.8
5	0.5	2.96	2.26
5	0.25	2.32	1.64[1]
5	0.12	1.82	1.52[2]
5	0.06	1.62[3]	—

[1] 1 negative. [2] 4 negatives. [3] 2 negatives.

When this work was begun, no international standard was available, but we were able to obtain from Dr. A. S. Parkes, 50 mg. of *LP.1*, a prolactin powder supplied by Riddle and Bates (1, 2), containing 10 Riddle-Bates units per mg. Since *LP.1* has been assayed by many different laboratories, it affords a temporary basis for comparing the reactivity of our pigeons with those of other investigators. The international standard preparation of lactogenic hormone was issued by the Department of Biological Standards, National Institute for Medical Research, Hampstead, London, in November, 1939. It was preserved in accordance with the instructions in the accompanying memorandum. Purified preparations of sheep and beef lactogenic hormone were made in essentially the same manner previously described (16, 17). Only those preparations with crop-stimulating potency equalling that of preparations shown to be homogeneous by solubility studies were used.

PROCEDURE AND RESULTS

Pigeon Assay of Lactogenic Hormone

Assay of LP.1 and purified lactogenic hormone. LP.1 and *L 250 S*, a preparation of sheep lactogenic hormone made in this laboratory, were compared in Silver King pigeons, 4 to 5 weeks from hatching and weighing 400 to 550 gm. Both preparations were dissolved in distilled water with the aid of a few drops of NaOH, and adjustment was made immediately to pH 7.6 with HCl. Dilutions were made with distilled water containing 2% butanol as a preservative. Four daily subcutaneous injections of 0.1 cc. were made in the tail region and necropsy was performed 24 hours after the last injection. Five birds were tested at each of several levels ranging from 0.06 to 1.0 mg., and in the range of the M.E.D., more were added. Only the reactive part of the crop was dissected, shaken clean of food (not washed) and weighed immediately. Crop-sac weights below 2 gm. have but little significance in judging hormonal potency since control crops may vary in weight from 1 to 2 gm. Nor should too much significance be attached to crop-sac weights above 4 gm., since in some birds, the heavier reactions show a tendency to slough as in normal 'crop-milk' formation, and this sequestrated tissue may be used as food. The crop weights obtained in response to the

TABLE 2. COMPARING THE EFFECTIVENESS OF LACTOGENIC PREPARATIONS *LP.1* AND *L 250 S* IN CAUSING MINIMAL CROP STIMULATION WHEN GIVEN SUBCUTANEOUSLY TO SILVER KING PIGEONS

Total dose in mg.	*L 250 S*			*LP.1*		
	No. of pigeons	+	−	No. of pigeons	+	−
0.25	—	—	—	10	9	1
0.12	20	20	—	13	4	9
0.06	20	7	13	5	0	5
0.03	20	3	17	—	—	—

TABLE 3. CROP RESPONSE IN SILVER KING PIGEONS TO DIFFERENT AMOUNTS OF INTERNATIONAL STANDARD LACTOGENIC HORMONE GIVEN IN 4 DAILY SUBCUTANEOUS INJECTIONS

Dose in mg.	No. of pigeons	Positive	Negative	Weight of crop-sacs, in grams
3.2	5	5	0	3.4
1.6	5	5	0	2.7
0.8	5	5	0	2.3
0.4	5	5	0	1.8
0.35	20	15	5	Control Wt.
0.2	20	7	13	Control Wt.

various levels of *LP.1* and *L 250 S* injected are given in table 1. It may be seen that the dose of *L 250 S* that was required to produce a given crop weight was approximately one half that of *LP.1*. In 1933 it was suggested (11) that one need not depend on the weight of the crop in assaying lactogenic hormone, and that a qualitative response should suffice since month-old control birds never show recognizable crop stimulation. McShan and Turner (18) have developed a curve based on the percentage of birds reacting to various levels of lactogenic hormone and use the level at which $50 \pm 11\%$ of 20 common pigeons respond as their unit. *L 250 S* was tested on 20 pigeons at each of 3 dose levels in the region of the M.E.D., but only enough *LP.1* was available for testing on 5 more pigeons at 0.25 mg., 8 more at 0.12 mg. and 5 at 0.06 mg. Table 2 contains the results obtained with the border-line doses of *LP.1* and *L 250 S*. The total dose of *LP.1* given subcutaneously for 4 days that could be expected to cause crop-sac stimulation in the majority of our pigeons is between 0.12 and 0.25 mg.[2] In the case of *L 250 S*, that dose is between 0.06 and 0.12 mg. As was found with the crop weight method of assay, this indicates that *L 250 S* has approximately double the crop-stimulating potency found in *LP.1*.

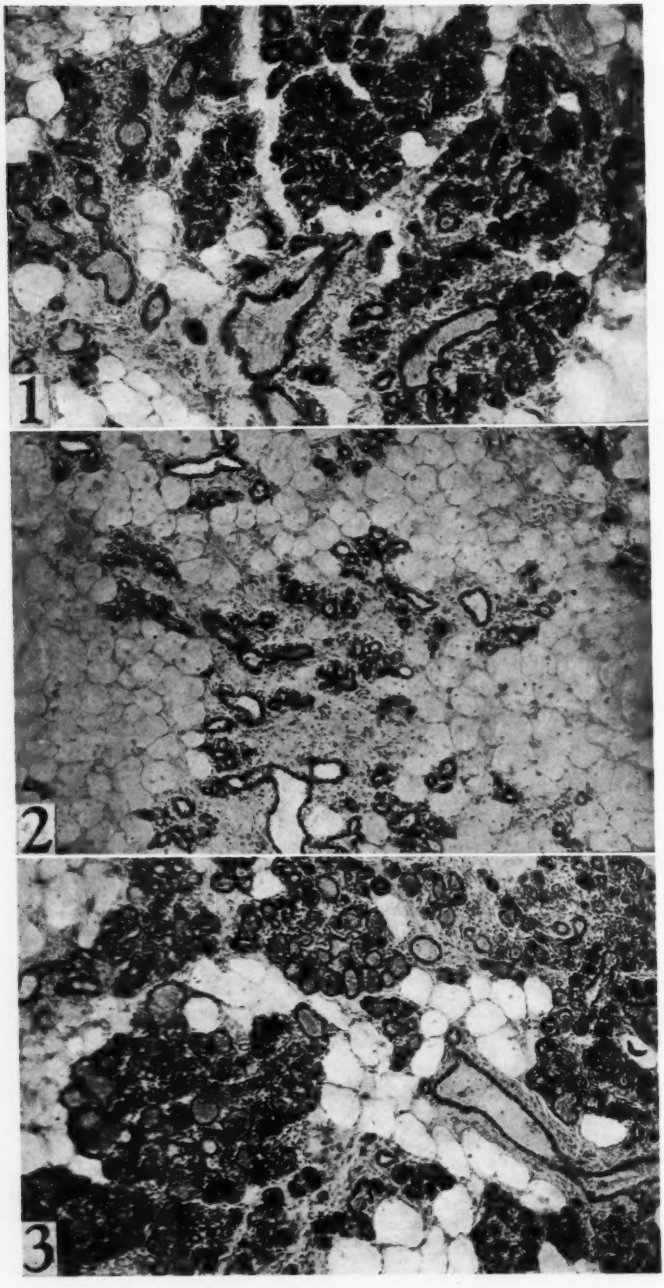
TABLE 4. RESPONSE OF GUINEA PIGS ON DIFFERENT DAYS OF THE ESTROUS CYCLE TO 4 DAILY INJECTIONS OF PURIFIED LACTOGENIC HORMONE

Day of cycle on which injections were begun	Total dose of hormone	No. of animals	Lactation	
			+	−
1–3	240 I.U.	16	10	6
4–8	240 I.U.	15	1	14
9–12	240 I.U.	16	0	16
13–16	240 I.U.	15	0	15

Assay of the international standard in pigeons. The international standard[3] was received in January, 1940, and was assayed in pigeons and guinea pigs. The 10 mg. tablets appeared yellowish and not white as purified lactogenic hormone should and, contrary to the statement in the accompanying memorandum, they were not readily soluble in water. A small amount of weak NaOH was used in dissolving the tablets at about PH 9.0 and the solution was then adjusted to PH 7.6 with HCl. Doses ranging from 0.2 to 3.2 mg. (2 to 32 I.U.) were given subcutaneously in the manner described above. Table 3 contains the results of these injections. It may be seen that the dose at which the majority of our pigeons may be expected to show crop-stimulation lies between 0.2 and 0.35 mg. (*LP.1* = 0.12–0.25 mg.; *L 250 S* = 0.06–0.12 mg.). A dose of

[2] Bergman, Meites and Turner (19) found that 12 of 20 common pigeons (60%) showed a positive crop reaction when injected intramuscularly with 0.15 mg. of *LP.1*. Intramuscular injections of lactogenic hormone have proven, in our hands, to be about one-half as efficacious as subcutaneous. Therefore, it may be assumed that the 300 gm. common pigeon used in Dr. Turner's laboratory shows minimal reaction to a smaller dose of hormone than our 450 gm. Silver King pigeons.

[3] The author is indebted to Dr. H. M. Evans and the Cutter Laboratories, Berkeley, for some of the international standard sent them by Dr. A. S. Parkes.



Figs. 1–3. See next page for legend.

0.2 mg. of the international standard as compared to 0.06 mg. of *L 250 S* was required to cause 7 of 20 (35%) positive crop reactions. Thus *L 250 S* was found to have about three times the crop-stimulating potency of the international standard.

Guinea Pig Assay of Lactogenic Hormone

Spontaneous lactation. When we (11) proposed the ovariectomized guinea pig as a suitable assay animal for lactogenic hormone, the objection was raised by Nelson (10) that too large a percentage of such animals lactated without any injections. That has not been observed in our colony, but the objection is recognized as a valid one. The disagreement may be due to colony variation or even to a difference of opinion as to what constitutes milk, colostrum or serous fluid. Unrecorded observations on a large number of normal, adult guinea pigs during all stages of the estrous cycle had led us to recognize that a negligible percentage of them lactated spontaneously—a few drops of milk could be expressed from the glands for usually not more than the first 3 days of the cycle, whereas in almost all, a clear or slightly turbid fluid (not white) could be expressed at estrus and for a day or so thereafter (see fig. 1 for histology). In a single experiment in which 54 animals were examined daily for spontaneous lactation during the first 5 days of the cycle, only one lactated. So for presumptive testing of substances for lactogenic activity, 5 animals per dose level have sufficed. For more exact determination of unitage not less than 20 animals have been used.

Influence of age. Although guinea pigs may be bred as early as the fourth month, and the mammary apparatus may be quite adequate for suckling purposes, two months later (i.e., at the end of pregnancy), the 6-month-old *nulliparous* guinea pig has a very small mammary apparatus. Some nulliparous animals, at two years of age, show very little mammary development over and above that seen in the average 6-month-old animal and they may even show less. It has been found that guinea pigs over 650 gm. in weight and 6 months of age that did not lactate in response to 8 mg. (240 I.U.) of purified lactogenic hormone usually had hypoplastic mammary glands (see fig. 2 for histology). These glands have been studied by serial section, by India ink injection and by means of the Spalteholz clearing technic. In the beginning of our study, in order to determine how young a guinea pig could be used, 49 tests were carried out on 26 estrous animals from 400 to 600 gm. in weight, and 4 to 5 months old. In each case, 4 mg. (120 I.U.) of purified lactogenic hormone were injected, and in only one instance—a 560 gm. animal—did lactation occur. As will be shown later, the majority of animals over 650 gm. lactate following this dose of hormone.

Influence of the stage of estrous cycle. In order to determine at what time during the estrous cycle the guinea pig would lactate most readily in response to lactogenic hormone, 61 animals were tested at various stages of the cycle with 8 mg. (240 I.U.) of purified hormone (4 daily subcutaneous doses of 2 mg.). The reaction was judged positive if on the fifth day, milk could be expressed from one or other of the mammary glands (see fig. 3 for histology of positive reaction). The results in table 4 show that in only one of 45 animals tested between days 4 and 16 was a positive response obtained, and that animal was in its fourth day of the cycle when injections were begun. Ten of 16 animals in which injections were started during the first three days of the cycle, lactated.

Fig. 1. MAMMARY GLAND OF AN UNTREATED, ADULT, VIRGIN GUINEA PIG, SACRIFICED ON THE FIFTH DAY OF THE ESTROUS CYCLE. Note serous secretion in some of the alveoli and ducts. A slightly turbid fluid was expressed from the nipple. Fig. 2. MAMMARY GLAND OF AN ADULT, VIRGIN GUINEA PIG, SACRIFICED ON THE FIFTH DAY OF THE ESTROUS CYCLE. No lactation followed the injection of 240 I.U. of lactogenic hormone. Note hypoplastic parenchyma. Fig. 3. MAMMARY GLAND OF AN ADULT, VIRGIN GUINEA PIG, SACRIFICED ON THE FIFTH DAY OF THE ESTROUS CYCLE. Lactation followed the injection of 240 I.U. of lactogenic hormone. Note milk in alveoli and ducts. A few mitoses were observed in parenchyma and stroma. All figures ×70.

The possibility that lactation may be initiated in guinea pigs in periods of the cycle other than one to three days after estrus by giving larger doses of lactogenic hormone has not yet been investigated. It will be noted that the period of decreased sensitivity to the hormone coincides rather closely with the diestrous phase of the cycle when new corpora lutea have formed in the ovary. In the latter part of the cycle, luteal degeneration takes place and the animal comes under the influence of the maturing follicular apparatus. The ovarian hormones, estrin and progestin, may counteract the injected as well as the animal's own lactogenic hormone. We were able to show that the mammary glands of nulliparous guinea pigs come under the influence of the animal's own lactogenic hormone cyclically (20). Thus, when unilateral mammectomy with or without ovariectomy was performed at various stages of the estrous cycle only those animals at estrus or one to two days later lactated spontaneously. This suggested a cyclic secretion of lactogenic hormone by the pituitary with a peak just after estrus and, incidentally, it precluded the taking of a control gland in guinea pig tests for that hormone. The occasional spontaneous lactation in normal, nulliparous guinea pigs and the frequent serous secretion also occur at this period. It would seem then, that any lactogenic hormone injected at this time would act with that of the animal's own pituitary in causing lactation. In all of the tests reported below, injections were begun not later than 24 hours after vaginal opening which occurs just prior to estrus in the guinea pig. This time can be extended to 48 hours without loss of accuracy.

Hypersensitiveness of guinea pigs to lactogenic hormone. In an earlier paper (11) on the guinea pig assay of lactogenic hormone, it was pointed out that anaphylaxis sometimes interfered with the repeated use of an animal through several estrous cycles. At one time contaminating proteins may have been partly responsible for this. However, it has been shown that our purified lactogenic hormone is a single protein (14, 15) and highly antigenic (21).

It has always been possible to demonstrate some degree of hypersensitiveness two weeks after a single injection of it in guinea pigs, either by anaphylaxis or skin reactions. Intracardial injection of 0.1 mg. (two weeks after sensitizing) invariably proved fatal, and doses as low as 0.01 mg. have also been lethal. Subcutaneous injections have been used in routine tests because the hormone is most effective when given by that route and because anaphylaxis occurs in only about 10% of our subcutaneous re-injections. Interdermal injections were found equally effective in causing lactation, but intradermal re-injections almost always caused hypersensitive skin reactions that varied from a slight edema to localized necrosis. It was observed that whenever a guinea pig showed a very severe skin reaction to the first of four daily intradermal injections of lactogenic hormone, the three subsequent injections caused reactions of decreasing severity. Dr. Maurice Priver (22) of Los Angeles has also observed considerable variation in the amount of local swelling following injections of our lactogenic hormone into women in the puerperium. He found that some women showed no local reaction to the same dose of the same preparation that caused a severe and painful swelling in others. It was possible to desensitize the hypersensitive individuals by gradually increasing the dose from 1 to 50 mg. Since excessive antihormonal substances should be listed among the possible causes for failure of women to lactate in the puerperium, a comparison of the skin reactions to lactogenic hormone in women who are lactating normally and those failing to lactate might be of interest. At first we were inclined to explain the skin reactions to lactogenic hormone on the basis of contaminating beef and sheep serum proteins and it is entirely possible that some of the severe reactions to this hormone reported in the literature may have been due to non-lactogenic proteins. However, by developing anti-sera against both beef and sheep sera and testing our beef and sheep lactogenic preparations with these anti-

sera, we were unable to demonstrate any such contaminations. Hypersensitiveness is developed toward the hormonal protein and it was found that equally severe anaphylactic or skin reactions occurred in guinea pigs sensitized to either beef or sheep lactogenic hormone when re-injected with either preparation.[4]

Assay of purified lactogenic hormone in guinea pigs. Experiments on 164 nulliparous guinea pigs between 650 and 1,000 gm. were carried out to determine the percentage response to 30, 60, 120 and 240 I.U. of lactogenic hormone. Table 5 contains the results. In each case listed, the animal was receiving an injection for the first time. The 120 I.U. level at which 56.6% of the animals lactated was then used to determine the reactivity of these guinea pigs to subsequent injections of the hormone. Excluding

TABLE 5. NUMBER OF GUINEA PIGS LACTATING FOLLOWING 4 DAILY SUBCUTANEOUS INJECTIONS OF DIFFERENT AMOUNTS OF PURIFIED LACTOGENIC HORMONE

Dose of Hormone		No. of Guinea Pigs	Lactation		% Positive
I.U.	mg.		+	−	
30	1	28	8	20	28.6
60	2	22	10	12	45.4
120	4	60	34	26	56.6
240	8	54	40	14	74.1

approximately 10% of the animals injected for the second series which died from anaphylaxis it was found that only 26 of 61 or 42.6% responded to 120 I.U. on the second test and 25 of 64 or 39.1% responded to the same dose upon being tested for the third time. This difference in response to first tests on the one hand and the second and third tests on the other seems to be a significant one. It may be possible to prove that some guinea pigs show a decreased mammary response to subsequent injections because of the development of antisubstances.

At any rate, this diminished responsiveness on the part of a group of guinea pigs suggested that some animals would probably not be suitable for repeated tests. In most bioassays involving repeated tests on the same animals, there is a tendency to eliminate the poor reactors or to stratify the animals in accordance with their mean reaction to oft-repeated tests with standard preparations. With this in mind, 63 tests made on 53 different guinea pigs that had previously lactated in response to not more than 240 I.U. of lactogenic hormone were analyzed. Each animal received 120 I.U. of the hormone in the usual manner and it was found that 38 of the 63 tests or 60.3% were positive.[5] This corresponds closely to the 56.6% positive reactions to 120 I.U. of hormone observed in the 60 first tests (table 5), and it is suggested that guinea pigs that have been proven reactive to not more than 240 I.U. would serve as well as, or better than animals being used for the first time. Variations in responsiveness of guinea pigs in different colonies can be expected, but if the international preparation is used to standardize each colony, this difficulty can be minimized. How long one may continue to use such 'proven' animals has not yet been determined. They have been found satisfactory from the time they were 6 months of age until they reached one and a half years. During this period the weight of such animals increases 50 to 100% and this should probably be considered in calculating dosage of hormone, although the variation in the response of individual guinea pigs over a period when their

[4] Solubility and amino-acid studies (15) show differences between beef and sheep lactogenic hormone, although the usual, so-called immunologic tests failed to do so. Quantitate precipitin tests by the Heidleberger technic have yet to be done.

[5] Although the comparison is not strictly valid, it was noted that when 68 tests were carried out by giving 120 I.U. of lactogenic hormone to 45 different animals which previously showed no response to 30 to 240 I.U. only 35.3% lactated.

TABLE 6. VARIABILITY IN LACTATIONAL RESPONSE IN INDIVIDUAL GUINEA PIGS TO SUBCUTANEOUS INJECTIONS OF PURIFIED LACTOGENIC HORMONE

Guinea Pig	Test 1		Test 2		Test 3		Test 4		Test 5	
	Dose I.U.	Reaction	Dose I.U.	Reaction	Dose I.U.	Reaction	Dose I.U.	Reaction	Dose I.U.	Reaction
1	30	−	60	−	120	−	120	−		
4	30	−	120	+	60	−	120	−	120	−
5	240	+	120	+	120	+	240	+		
8	240	+	60	−	120	−	240	+		
9	240	+	120	+	60	−	240	+	240	+
10	240	+	30	−	120	+	240	+		
12	120	+	60	−	120	+	240	+	120	−
15	60	+	30	+	120	+	240	+	120	+
17	60	+	30	+	120	+	120	+		
19	120	+	60	+	120	−	240	+	120	−
20	60	−	120	+	60	−	30	−	120	+
21	60	−	120	+	60	+	120	−		
22	120	+	60	+	120	+	240	+	120	+
29	60	+	30	−	120	+	240	+		
37	60	−	120	+	120	+	240	+		
39	60	+	30	−	120	−	240	−		
44	120	−	120	+	120	+	240	−		
45	60	−	120	+	30	−	120	−	240	+
46	120	−	120	+	120	+	240	−		
49	60	−	120	+	120	+	240	−		
58	120	+	60	−	240	+	120	+	240	+
61	60	+	30	−	120	−	120	−		
77	120	−	120	+	240	+	120	+	60	+
79	120	+	60	+	240	+	120	−	240	+
81	240	+	120	+	120	+	240	−		
82	60	+	120	+	30	−	240	−		
85	120	+	60	−	120	+	240	+	60	−
88	120	+	60	−	120	+	240	+	120	+
95	30	+	30	−	60	−	240	+		
96	60	+	30	−	120	+	240	−		
116	120	+	60	−	120	−	240	+		
140	30	−	60	+	120	+	30	−	120	−
154	120	+	120	+	60	−	240	+	120	+
180	60	−	60	−	60	−	60	−		
189	30	−	60	+	60	+	120	−	240	+
190	240	+	60	+	30	+	30	−		
198	120	+	60	+	60	+	60	+	30	−
212	60	+	60	−	120	+	120	−		
218	30	−	120	+	60	−	120	+	240	+
157	120	+	60	−	60	−	240	−		
159	120	+	60	+	120	+	120	+		
150	120	+	60	−	60	−	240	−		
148	120	+	60	+	120	+	240	−		
147	240	+	120	+	120	+	240	−		
146	30	−	120	+	120	−	120	−		

weight has changed but little was found to be several hundred per cent. In table 6 are listed the reactions of 45 guinea pigs, which have been used at least four times for assays of purified lactogenic hormone containing approximately 30 I.U. per mg. as determined by the crop response in pigeons. Of the 45 animals 21 showed no discrepancy in their responses to the hormone while most of the remaining 24 showed a variation of 100 to 200%. This variation is not greater than that usually experienced in most bioassays, and it would be difficult to obviate since the factors influencing it are unquestionably multiple.

Assay of the international standard in guinea pigs. When the international standard of lactogenic hormone was assayed on pigeons, parallel tests were carried out on guinea pigs previously found responsive to not more than 240 I.U. of purified hor-

mone. Only two levels of the international standard were tested because the supply was limited. Total doses of 4 mg. (40 I.U.) and 8 mg. (80 I.U.) were given in four equal daily subcutaneous doses on the first four days of the estrous cycle. A test was considered positive if the animal lactated within 24 hours of the last injection. At the 4 mg. dose, 7 of 20 (35%) lactated, and at the 8 mg. dose, 11 of 20 (55%) lactated. If the guinea pig M.E.D. be considered the smallest amount of lactogenic hormone causing lactation in the majority of at least 20 animals,[6] it would follow that it approximates 8 mg. or 80 I.U.[7] Since we have shown that 60.3% of 'proven' guinea pigs lactated when injected with 4 mg. of the purified hormone, it would appear that by the guinea pig assay that preparation has about twice the potency of the international standard. By the pigeon test it was about three times as potent.

DISCUSSION

Purified lactogenic hormone was found to be about twice as effective as the international standard in causing lactation in the virgin guinea pig and about three times as effective in causing crop-sac stimulation in pigeons. This difference may be due to the use of an insufficient number of animals for accurate comparison, but it should also be remembered that the present international standard is not a pure substance. Impurities present in it might conceivably synergize with lactogenic hormone in its mammary-stimulating, but not in its crop-sac-stimulating action. Or they might act as absorption-retarding agents to different degrees in the two species. Such questions could readily be answered by experimentation, but it would be more reasonable to ask that future contributions towards the standard be thoroughly assayed and, if necessary, rendered free of any of the other recognizable pituitary factors, blood proteins, etc. Finally, they should be shown to be essentially pure by use of the solubility test. No implication is intended that the present standard is not a useful and valuable one. On the contrary, it has provided a very necessary common denominator for investigators in different laboratories to use in comparing crop-sac-stimulating activity. No claim has been made that it could be used for other purposes having to do with a thorough understanding of the functions of the isolated hormone.

The possibility that other hormones present in crude pituitary extracts (or in the international standard) may enhance the activity of the purified lactogenic hormone requires some consideration. Because thyroxin and thyrotropic hormone increased the milk yield of lactating cows, and because crude pituitary extracts also did this in a way that made it seem unlikely that the crop-sac-stimulating hormone was responsible for the whole effect, Folley and Young (23, 24) questioned the wisdom of restricting the term 'lactation hormone' to a single pituitary substance. Several other investigators have also studied the effect of pituitary extracts on animals already lactating, but the increase in milk yield of such animals has never been generally accepted as a criterion by which to judge the efficacy of the lactogenic hormone. It might be misleading to do so. One usually associates the function of increasing milk-output in a lactating animal with the term galactogogue and it might be said to be subserved by other hormones such as thyroxin, thyrotropin or even by dietary factors. That such non-specific lactation-influencing substances might have some effect on the outcome of tests on animals which after all, are "on the verge of lactating" in response to their own lactogenic hormone is conceivable and can be investigated by employing a standardized animal such as used in this work and purified substances suspected of syner-

[6] Nulliparous guinea pigs not less than 650 gm. in weight being used for the first time or preferably animals that have previously lactated following the injection of not more than 240 I.U. of lactogenic hormone.

[7] According to Bergman et al. (19) approximately 52 mg. of *LP.1* were required to cause lactation by their rabbit test. Calculations indicate that slightly more of the international standard would be required for this.

gizing with the lactogenic hormone. Regardless of the inadequacies of the various terms used to designate the hypophyseal crop or mammary-stimulating hormone (lactogenic, lactogen, prolactin, galactin, mammotropin) it would seem better to associate such names with a single substance that initiates in the estrin or estrin-progestin-prepared mammary gland, a functional alveolar growth leading to lactation.

SUMMARY

A comparison of hypophyseal lactogenic preparations has been made in pigeons and guinea pigs. The international standard of lactogenic hormone was found to be about one-third, and *LP.1* about one-half, as potent as the purified hormone *L 250 S* when compared by either the crop-weight response or the minimal crop reaction in pigeons. The approximate M.E.D. for the three preparations were: *I.S.*, 0.2 to 0.35 mg.; *LP.1*, 0.12 to 0.25 mg.; *L 250 S*, 0.06 to 0.12 mg.

Nulliparous guinea pigs, one-half to one and a half years old, weighing over 650 gm. injected subcutaneously on days one to four of the estrous cycle, were also used in comparing the international standard with this laboratory's purified lactogenic hormone. Animals under 650 gm. (5 to 6 months old), and those in which injections were begun on days other than one to four of the cycle did not respond well enough to lactogenic hormone to warrant their use. Acceptable animals were used repeatedly through several estrous cycles provided fatal anaphylaxis did not intervene. Of 60 guinea pigs injected for the first time with 4 mg. (120 I.U.) of purified lactogenic hormone, 34 (56.6%) lactated on their first test. Of a standardized group of 63 guinea pigs (proven reactive to not more than 240 I.U. of lactogenic hormone) 38 (60.36%) lactated following injection of 120 I.U. of purified hormone. The international standard when tested on guinea pigs that had been proven reactive caused lactation in 7 of 20 (35%) at the 4 mg. (40 I.U.) dose and 11 of 20 (55%) at the 8 mg. (80 I.U.) dose. Thus the international standard was found to be about one half as potent as purified lactogenic hormone by the guinea pig assay.

REFERENCES

1. *Bull. Health Org.* 7: 887. 1938.
2. *Bull. Health Org.* 8: 909. 1939.
3. STRICKER, P., AND F. GRÜTER: *Compt. rend. soc. de Biol.* 99: 1978. 1928.
4. TURNER, C. W., AND W. U. GARDNER: *Mo. Agr. Exp. Sta. Res. Bull.*, 158, 1931.
5. GARDNER, W. U., AND C. W. TURNER: *Mo. Agr. Exp. Sta. Bull.* 196, 1933.
6. CORNER, G. W.: *Am. J. Physiol.* 95: 43. 1930.
7. ASDELL, S. A.: *Cornell Veterinarian* 21: 147. 1931.
8. LYONS, W. R., AND H. R. CATCHPOLE: *Proc. Soc. Exper. Biol. & Med.* 31: 305. 1933.
9. NELSON, W. O., AND J. J. PFIFFNER: *Proc. Soc. Exper. Biol. & Med.* 28: 1. 1930.
10. NELSON, W. O.: *Anat. Rec.* 60: 69. 1934.
11. LYONS, W. R., AND H. R. CATCHPOLE: *Proc. Soc. Exper. Biol. & Med.* 31: 299. 1933.
12. RIDDLE, O., AND P. F. BAUCHER: *Am. J. Physiol.* 97: 617. 31.
13. RIDDLE, O., R. W. BATES AND S. W. DYKESHORN: *Am. J. Physiol.* 105: 191. 1933.
14. LI, C. H., W. R. LYONS AND H. M. EVANS: *Science* 90: 622. 1939.
15. LI, C. H., W. R. LYONS AND H. M. EVANS: In press.
16. LYONS, W. R.: *Proc. Soc. Exper. Biol. & Med.* 35: 645. 1937.
17. LYONS, W. R.: *Cold Spring Harbor Symp. Quant. Biol.* 5: 198. 1937.
18. MCSHAN, W. H., AND C. W. TURNER: *Proc. Soc. Exper. Biol. & Med.* 34: 50. 1936.
19. BERGMAN, A. J., J. MEITES AND C. W. TURNER: *Endocrinology* 26: 716. 1940.
20. LYONS, W. R.: *Anat. Rec.* Suppl. 58: 76. 1934.
21. BISCHOFF, H. W., AND W. R. LYONS: *Endocrinology* 25: 17. 1939.
22. PRIVER, M.: Personal communication.
23. FOLLEY, S. F., AND F. G. YOUNG: *Proc. Roy. Soc. London, s.B.* 126: 45. 1938.
24. FOLLEY, S. F., AND F. G. YOUNG: *Biochem. J.* 33: 192. 1939.

EFFECTS OF ANTERIOR PITUITARY PREPARATIONS IN EXPERIMENTAL PANCREATIC DIABETES

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WHETHER OR NOT the diabetogenic effects of anterior pituitary preparations and the effect upon growth are related has interested several observers. Shipley and Long (1) and Young (2) found that fractions which had the greatest effect upon growth were also the most diabetogenic. Young (3) considered the possibility that only one pituitary factor is involved, and that the response of the pancreas to it could determine whether nitrogen storage or diabetogenic effects would result. In this connection he cited Mirsky's experiments (4), which suggest that growth preparations may influence protein metabolism by way of the pancreas.

Several studies in this laboratory have dealt with the metabolic effects of anterior pituitary growth preparations in normal, phlorhizinized, and depancreatized bitches. Single large injections were given to fed animals. The normal ones showed only "growth effects," for example, rise in weight and marked nitrogen storage. Glycosuria and ketonuria did not occur, and oxidation of carbohydrate was not evidently impaired (5). In depancreatized animals, treated with insulin, the same dose of this growth preparation produced the diabetogenic effects reported by others (6), namely hyperglycemia, glycosuria, ketonuria and lipemia. In meat fed phlorhizinized animals the existing glycosuria was diminished, in parallel with protein metabolism, and no evidence for glucogenesis from fat presented itself (7). It seemed possible that the diabetogenic effects were prevented in normal and phlorhizinized animals by increased secretion of insulin. Such an assumption did not simultaneously account for the nitrogen storage produced in normal animals, for this could not be induced by injecting insulin.

To test this idea further, we have performed experiments in which the insulin dosage of depancreatized animals was increased when the growth preparation was injected. The results were compared with those which the growth preparation alone produced in normal animals. We also determined whether the effects of the growth preparation on weight and nitrogen storage could be imitated by giving normal animals an amount of sucrose which, in depancreatized animals, simulated the effects of the growth preparation on glycosuria and hyperglycemia.

EXPERIMENTAL CONDITIONS

The analytical methods are cited in a preceding publication (7). The animals were adult bitches, whose diets had been adjusted previously so that weight was maintained or slowly increased and no food was refused. Urine collection periods were terminated by catheterizing and washing. Nitrogen balances were continuous, and included analyses of food, urine and stools.

Three diets were used. *Diet 1* consisted of beef heart, cracker meal and bone meal. Constancy was maintained by the method used with *diet 8*. *Diet 5* was a dry mixture of casein, cracker meal, corn oil, yeast and Karr's salt mixture, to which a measured amount of water was added before feeding. *Diet 8*, for depancreatized animals, was prepared as follows: 8150 gm. of beef heart trimmed free of fat, 1350 gm. of trimmed fresh pancreas, 1500 gm. of casein, 100 gm. of Karr's salt mixture and 350 gm. of dibasic calcium phosphate were mixed in a mechanical dough mixer until

uniform. The product was put up in 300 gm. packages, which were wrapped in heavy paraffined paper, frozen, and thawed out as needed. Sample packages were partly hydrolyzed at room temperature with concentrated hydrochloric acid, and after 3 to 4 days the resulting fine suspension was analyzed for nitrogen. All animals received 15 drops of haliver oil daily.

Depancreatized animals received their food and insulin in equal portions at 8 A.M. and 5 P.M. In the experiments shown in figures 1, 2 and 3 the protamine zinc

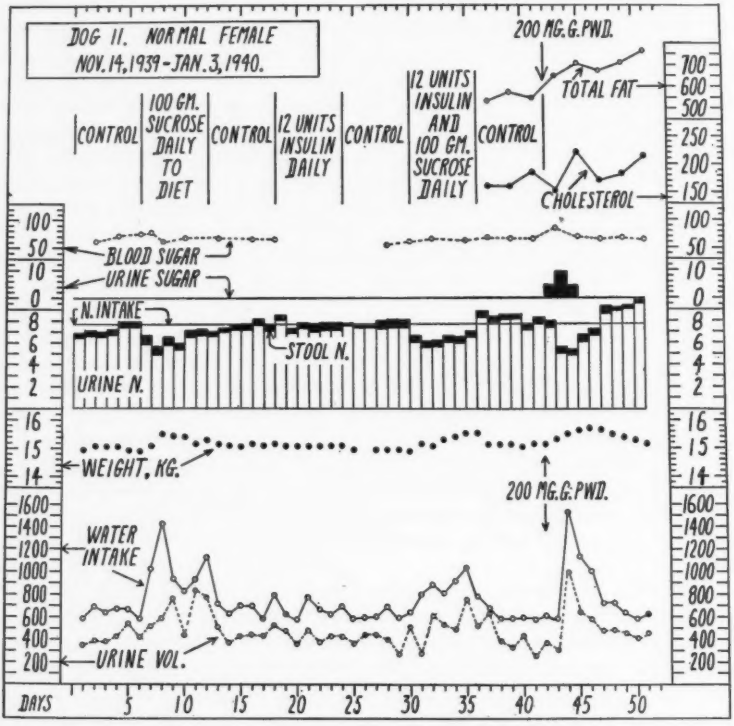

Fig. 1. Addition of sucrose to the diet of a normal animal produced effects on water intake, weight and nitrogen balance similar to those of the growth preparation (200 mg. G. PWD.). Insulin did not produce these effects or augment those of sucrose.

insulin of Eli Lilly & Company was used. The commercial insulin of the same firm was used in the other experiments. Antuitrin-G, obtained from Parke, Davis & Company, was used in the experiments shown in figure 4. In all other experiments a dry powder supplied by the same firm was used. This growth preparation originated from beef anterior lobes, and in its purification the gain in weight of normal and hypophysectomized rats was the criterion of potency. The powder was dissolved in 0.01 N sodium hydroxide; the solution was neutralized until precipitation just began, then clarified by centrifugation and injected. The same powder had been tested in a phlorhizinized animal (7).

That pancreatectomies were as complete as possible was verified in two ways. Withdrawal of insulin, before experiments began, produced D:N ratios of 2.9 in

the meat fed animals (fig 4), and autopsies after termination of experiments disclosed no pancreatic tissue on gross inspection.

Blood specimens were taken before the morning feeding, hence the figures for non-protein nitrogen and total fat are postabsorptive values. The blood sugar values represent postabsorptive true sugar.

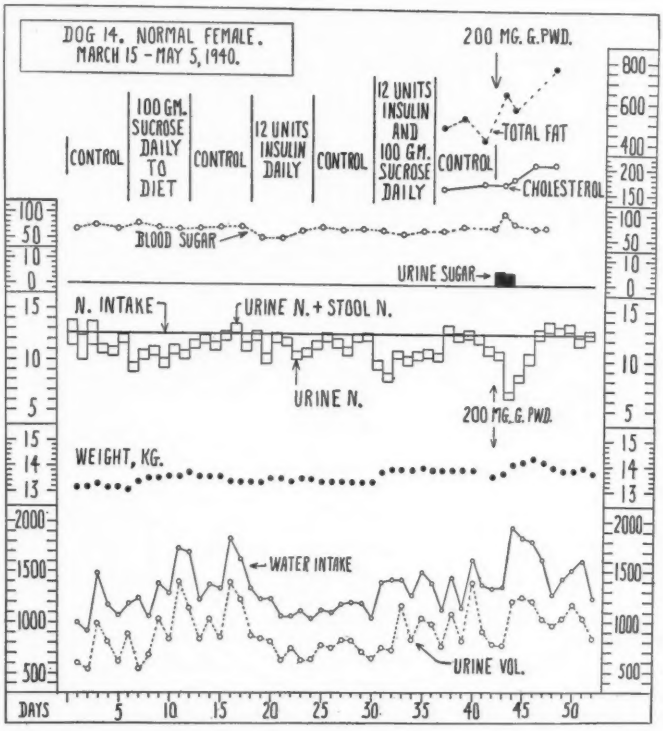

Fig. 2. The results shown in figure 1 are confirmed in a different animal receiving a different diet.

RESULTS

Normal dog 11. During the first 42 days we observed the effects of adding sucrose to the diet, of injecting insulin without changing the diet, and of injecting insulin while adding sucrose to the food (fig. 1). Following this, an experiment with 200 mg. of the growth preparation was performed. The urine nitrogen is shown in the bar graph, and superimposed upon it (in black) is the stool nitrogen. When the sum falls below the line showing the nitrogen intake the balance is positive. The water intake included water present in the food, found by moisture determinations; and water available by oxidation, calculated by means of factors (8) from analyses of the diet for protein, fat and carbohydrate. Water available from 100 gm. of sucrose added to the diet was not included in the intake. *Diet 1* was fed.

The effects of the growth preparation are shown clearly. The rise in weight was soon lost again, and was accompanied by a rise in water intake exceeding the rise in urine volume. The urine nitrogen fell sharply, and a positive balance resulted. These

changes also followed addition of 100 gm. of sucrose to the daily ration. They were not produced by two daily injections of insulin which totalled 12 U, and were not more marked in the experiment with insulin and sucrose than in the one with sucrose alone. In experiments mentioned in an earlier paper (7) 3 10-unit doses of insulin were given daily for 2 days, each accompanied by intravenous injection of 16.6 gm. of glucose, and no effect on nitrogen output resulted.

In the present instance we observed, for the first time in a normal animal, a slight

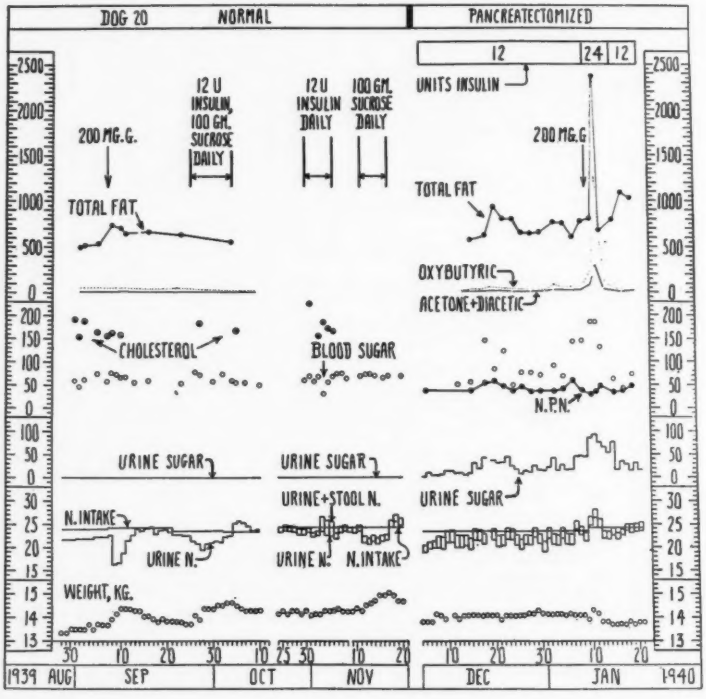

Fig. 3. The effects of the growth preparation (200 mg. G.) on Jan. 9, 1940, after pancreatectomy, were practically the reverse of those obtained with the same dose of this preparation in the normal animal, on Sept. 7, 1939.

glycosuria on the 3 days following injection of the growth preparation. The glucose output was 4.6, 9.4 and 4.6 gm. on these days.

Normal dog 14. The same experiments were repeated, using a different animal and *diet 5* (fig. 2). While the urine nitrogen fell in each experiment, the effect of the growth preparation thereon was most striking. When calculated in 6-day periods, the improvement in the nitrogen balance, in grams per day, was as follows: sucrose alone, 2.49; insulin alone, 0.64; sucrose and insulin, 1.88; growth preparation, 3.06. During 2 days after injection of the growth preparation 5.5 and 4.7 gm. of glucose were excreted. The true sugar content of the blood rose to 100 for one day, and the total fat increased slightly. Acetone, diacetic acid, and oxybutyric acid were determined quantitatively in the urine and remained unchanged.

Dog 20. These observations were made before and after pancreatectomy (fig. 3). In the normal animal, 200 mg. of the growth preparation caused a slight rise in blood fat, no ketonuria, no change in the postabsorptive true sugar content of the blood,

no glycosuria, and the usual marked fall in urine nitrogen and increase in weight. After pancreatectomy the same dose of this powder produced a lipemia, hyperglycemia, ketonuria, and increase in glycosuria with some loss of nitrogen, in spite of the increase of 12 units in the daily dose of insulin. The experiments with insulin plus sucrose, insulin alone, and sucrose alone, in the normal animal, confirm those already presented.

Dog 16. These experiments (fig. 4) were important in establishing conditions for those presented in figures 3 and 5. The animal was pancreatectomized after it had

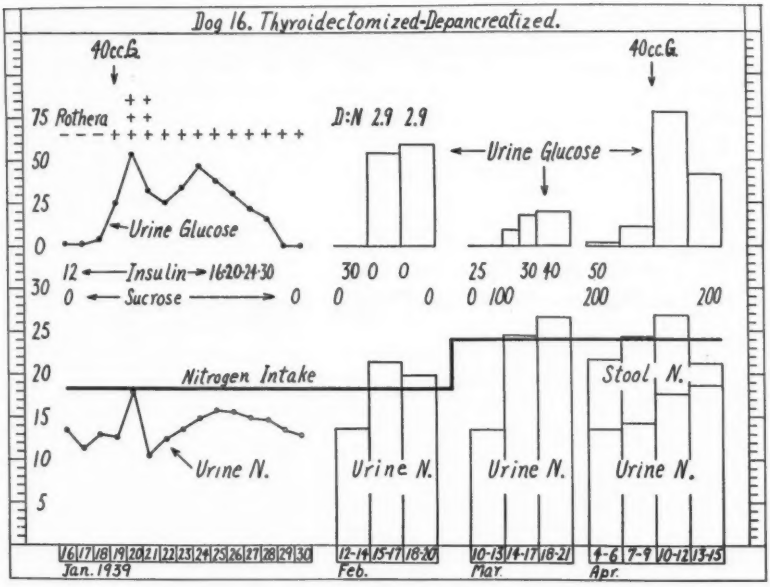

Fig. 4. The pituitary preparation (40 cc. G.) produced ketosis and glycosuria in an insulin-treated depancreatized animal in the absence of the thyroid. The parallelism between glucose and nitrogen excretion in all experiments is partly referable to changes in absorption (see text).

been thyroidectomized and used in experiments with phlorhizin (7). No parathormone was required to maintain the serum calcium. At first a diet containing beef heart, pancreas, agar and salt mixture was used. Where the rise in nitrogen intake is shown this diet was replaced by one which differed from *diet 8* only in the use of bone ash instead of dibasic calcium phosphate. Neither of these diets was satisfactory. The urine nitrogen was far below the intake. The stools were highly offensive. The urine nitrogen rose and the stools promptly became normal when hyperglycemia and glycosuria were produced, whether by giving antuitrin-G, by withdrawing insulin, or by giving insulin and an excess of sucrose. The suggestive parallelism between nitrogen and glucose in the urine in the first experiment with the growth preparation was therefore discounted as possibly due to changes in absorption. When *diet 8* was used (fig. 3, 5) the stools of depancreatized animals receiving 24 gm. of nitrogen per day were normal as to odor, easy to collect, and contained on an average 2.2 gm. of nitrogen per day. The observed fluctuations were of no consequence.

Experiments shown in the preceding figure were continued, using the same thyroidectomized depancreatized animal (fig. 5). No infection occurred during any

experiment, and anorexia only during the last one. In the first experiment 200 mg. of the growth preparation was given, and the insulin dosage was simultaneously changed from 12 to 24 U per day. This produced some of the results seen in intact animals—a rise in weight subsequently lost again, a definite fall in urine nitrogen, a positive nitrogen balance, and a fall in blood non-protein nitrogen. But the hyperglycemia and increase in glycosuria persisted, in spite of diminished catabolism of protein.

After 3 days the insulin dosage was decreased to 18 U daily, for the next 20 days. A return to the control level of 12 U caused increased glucose excretion, but after

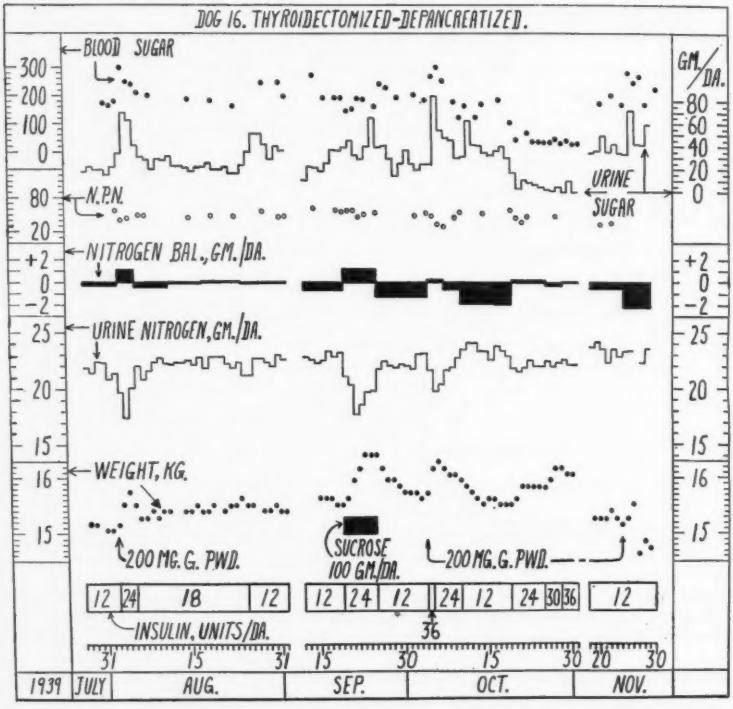

Fig. 5. Injecting the growth preparation (200 mg. G. PWD.) and simultaneously increasing the insulin dosage caused a gain in weight and nitrogen storage in a thyroidectomized depancreatized animal in spite of a large increase in the glycosuria.

25 additional days at this dosage the original control level of glycosuria was reached again. Adding 100 gm. of sucrose to the daily ration and raising the insulin dosage to 24 U per day produced effects qualitatively like those of the growth preparation and the same amount of insulin. The sucrose caused a larger increase in the weight and nitrogen balance, and a smaller increase in blood sugar and glucose excretion. A second experiment with 200 mg. of the growth preparation was then performed, the insulin dosage being increased to 36 U on the day when the growth preparation was injected, and to 24 U on the 5 following days. The results confirmed those of the first experiment. The glycosuria remaining from this experiment was abolished by progressively increasing the insulin dosage. A final experiment in which the

growth preparation was injected without a simultaneous increase in insulin dosage had no effect on nitrogen output on the second day following the injection, when positive results are always greatest. On this day the glucose output increased 33.6 gm. The next day refusal of half the ration complicated further observations.

DISCUSSION

The experiments in which we doubled or trebled the insulin dosage of depancreatized animals on the day when the growth preparation was injected were not complicated by anorexia, vomiting, or infection, so that conditions were favorable for metabolic studies.

It is evident that in the thyroidectomized depancreatized animal (fig. 5) the growth preparation and additional insulin produced nitrogen storage. Greater utilization of carbohydrate was not the cause of this, for the glycosuria increased markedly. If the additional insulin alone had produced the nitrogen storage by stimulating protein synthesis directly (9), one might have expected evidence of this direct effect in normal animals, and perhaps a summation of it with the indirect sparing effect of sucrose. Neither of these results was obtained. Nor was inhibition of the diabetogenic effect of the growth preparation by insulin (7) at all obvious, but we shall return to this point after considering the source of the glycosuria which this pituitary preparation caused in our depancreatized animals.

Could the pituitary preparation have produced nitrogen storage in normal animals and diabetogenic effects in depancreatized ones by causing extensive conversion of fat to glucose? In normal animals the addition of 100 gm. of sucrose to the daily ration produced increases in weight and water intake, and nitrogen storage, without glycosuria or postabsorptive hyperglycemia. In the thyroidectomized depancreatized animal (fig. 5) sucrose, supplemented with the same amount of insulin as the pituitary preparation, produced nitrogen storage, hyperglycemia, and an increase in glycosuria. But one would have to believe that the pituitary preparation caused extensive formation of glucose from fat only in normal and depancreatized animals, not in phlorhizinized ones (7), to accept this point of view.

Diet 8 was practically carbohydrate-free. The entire glycogen store, which diabetogenic preparations may diminish, leave unaltered, or increase (2, 10) would not suffice to explain the glycosuria. During 6 days after injection of the growth preparation the total increase in glucose output of *dog 20*, weighing 14.1 kg., amounted to 261 gm., although 72 additional units of insulin were given; *dog 16*, weighing 15.1 kg., showed a total increase in glucose output of 189 gm. during 12 days following the injection, although it received 90 additional units of insulin during this time.

Increased conversion of protein to glucose probably occurred in one experiment shown at the end of figure 3. The nitrogen output for 3 days after injection of the growth preparation averaged 2.54 gm. per day more than during the 3 preceding days. Using the D:N ratio of 2.8, this would account for 7.1 gm. of additional glucose, the output of which, however, rose 40.7 gm. per day. In all other experiments the nitrogen output fell, so that additional glucose from protein was not a factor at all.

Interference with utilization of glucose formed from protein is the only adequate explanation of the glycosuria. At the height of glucose excretion, on the second day following injections of the pituitary preparation, the D:N ratios in 4 experiments shown in figures 3 and 5 were 3.52, 3.96, 4.25 and 3.08. In 3 of these experiments the average ratio for the first 3 days after injections was 2.96, and for the second 3 days, 2.08. Attributing the glycosuria to this cause also accounts for the fact that in meat fed phlorhizinized animals, which were already excreting the glucose formed

from protein, this pituitary preparation did not increase the glycosuria, but diminished it in parallel with its effect on urine nitrogen (7).

Since no impairment of utilization of carbohydrate was observed in normal animals after large single injections of the growth preparation, the pancreas apparently prevented this diabetogenic effect. Our failure to prevent it in depancreatized animals by doubling or trebling the maintenance dose of insulin may suggest either that the increase of insulin output in intact animals is very large, or that presence of the pancreas modifies the situation in other respects. Since repeated injections of diabetogenic preparations produced marked changes in the islets (11, 12), some of the injected material may be retained there.

Glycosuria produced with diabetogenic preparations is attributed to increased conversion of protein to glucose and to interference with utilization of carbohydrate (13). If this view is correct, our results indicate that insulin prevents the first of these effects more readily than the second. In normal and phlorhizinized animals the pituitary preparation which we used diminished nitrogen output; when supplemented with insulin it also produced this effect in the depancreatized thyroidectomized animal, even though the glycosuria was greatly increased.

SUMMARY

In normal bitches receiving constant diets single large injections of an anterior pituitary preparation caused increases in water intake and weight, storage of nitrogen, a slight rise in blood fat, negligible glycosuria, and no ketonuria. The positive effects on weight and nitrogen storage could be imitated by adding 100 gm. of sucrose to the daily ration; they could, however, not be traced to overproduction of glucose from fat, since no indisputable evidence for this was seen when the pituitary preparation was administered to phlorhizinized or depancreatized animals.

In fed, insulin treated, depancreatized animals the same pituitary preparation caused lipemia, ketonuria, hyperglycemia, an increase in glycosuria, and some loss of nitrogen. On high protein, carbohydrate-free diets, the increase in glycosuria was greater than could be accounted for by glycogenolysis, and occurred even when nitrogen excretion was diminished. The D:N ratios permitted the assumption that the increase in glycosuria was caused by interference with utilization of glucose formed from protein.

In a thyroidectomized depancreatized animal nitrogen storage was regularly produced by injecting the pituitary preparation and simultaneously increasing the insulin dosage 12 U. This amount of insulin did not prevent the increase in glycosuria or other diabetogenic effects of the pituitary preparation, and in normal animals it did not produce significant nitrogen storage or augment the nitrogen storage produced with sucrose. An increase in insulin output is apparently not the immediate and only cause of the nitrogen storage produced by the growth preparation, but may be an essential condition for it.

REFERENCES

1. Shipley, R. A., and C. N. H. Long: *Biochem. J.* 32: 2242. 1938.
2. Young, F. G.: *J. Endocrinol.* 1: 339. 1939.
3. Young, F. G.: *Brit. M. J.* 2: 393. 1939.
4. Mirsky, I. A.: *Endocrinology* 25: 52. 1939.
5. Gaebler, O. H.: *Am. J. Physiol.* 110: 584. 1935.
6. Houssay, B. A., A. Biasotti, and C. T. Rietti: *Compt. rend. Soc. de biol.* 111: 479. 1932.
7. Gaebler, O. H., and W. J. Zimmerman: *Am. J. Physiol.* 128: 111. 1939.
8. Adolph, E. F.: *Physiol. Rev.* 13: 336. 1933.
9. Mirsky, I. A.: *Am. J. Physiol.* 124: 569. 1938.
10. Houssay, B. A., A. Biasotti and R. G. Dambrosi: *Compt. rend. Soc. de biol.* 125: 542. 1937.
11. Richardson, K. C., and F. G. Young: *J. Physiol.* 91: 352. 1937.
12. Richarson, K. C., and F. G. Young: *Lancet* 1: 1098. 1938.
13. Long, C. N. H.: *Proc. Assoc. Research in Nervous and Mental Disease* 17: 284. 1936.

INCREASED FAT AND DECREASED CARBOHYDRATE APPETITE OF PANCREATECTOMIZED RATS

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BERNARD, who in 1859 first stated his concept of the constancy of the internal environment, described various physiological mechanisms, part responses of the organism, responses of individual organs, which contribute to the maintenance of this constancy. We have recently found that behavior mechanisms, responses of the total organism, may also serve to maintain the constancy of the internal environment. The existence of these behavior mechanisms became established in experiments in which certain physiological mechanisms had been excluded. Thus, after adrenalectomy had removed the chief physiological means of regulating sodium metabolism, it was found that the animal itself made an effort to maintain the sodium balance by seeking and ingesting large amounts of sodium chloride (1). Similarly, after parathyroidectomy had removed the physiological mechanisms for the maintenance of a constant calcium balance, the animals themselves made an effort to correct the calcium loss by ingesting large amounts of calcium solution (2). Thus, the dietary selections made by these animals disclosed the deficiencies and needs of the animals. This technic has been applied to the study of the needs of pregnant and lactating rats, rats with vitamin A and B deficiency, and other normal and pathological conditions (3, 4).

A study has now been made of the dietary selections of pancreatectomized rats. We were interested to learn whether pancreatectomized rats seek fat or carbohydrate and whether their selections actually help to maintain the constancy of their internal environment.

METHODS

The cages used for these experiments consisted of a revolving drum with a cyclometer and a detachable living compartment. Two types of living compartments were used in different parts of the experiments. One compartment was 11 × 3 × 5¼ inches and contained a cup for food and a graduated inverted bottle for water. The food (standard McCollum diet) was made according to the following formula.

Graham flour	725 gm.
Skim milk powder	100 gm.
Casein	100 gm.
Butter	50 gm.
Calcium carbonate	15 gm.
Salt	10 gm.

Of this diet carbohydrate constituted 59.2%; fat, 14.1%; and protein, 26.7%. The other compartment was 9¼ × 9¼ × 5¼ inches and contained 3 food cups and 8 graduated inverted bottles. In the control experiments 11 receptacles were used. They contained the following substances.

Received for publication August 2, 1940.

[1] Henry Dennison Scholar—1939–1940.

Food cups for solids	*Bottles for fluids*
	Olive oil
	Cod liver oil
Casein (autoclaved and purified)	Sodium chloride.....3%
Sucrose	Dibasic sodium phosphate....8%
Dried baker's yeast or liver powder	Calcium lactate.....2.4%
	Potassium chloride.....1%
	Magnesium chloride.....0.5%
	Tap water

Records were made daily of activity, of intake of the various substances, of vaginal smears, and weekly of body weight. Blood sugar determinations were made only at infrequent inter-

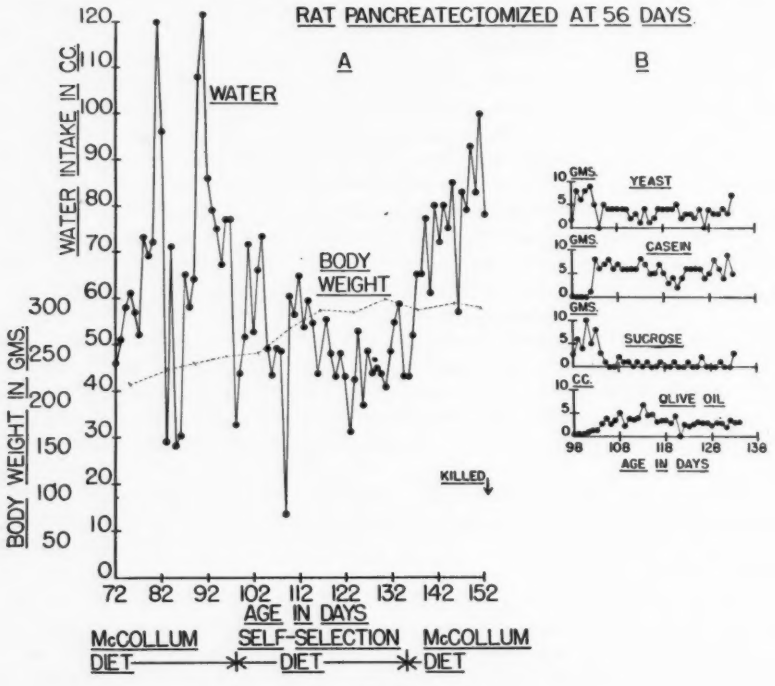

Fig. 1

vals because the necessary period of starvation and the removal of the blood greatly disturbed the food and fluid intake and activity.

The experiments were conducted in 2 series. In the first series we used 7 rats which had been pancreatectomized at an early age and on the standard McCollum diet had consistently shown marked symptoms of diabetes, a high water intake, an increased food intake, hyperglycemia, and reduced rate of growth. These rats were permitted to select their own diet from the substances enumerated above for periods which ranged from 36 to 202 days and then were returned to the standard McCollum diet. In the second series 8 normal rats were pancreatectomized when they had been on the self-selection diet for at least 40 days. Twenty-six to 152 days after pancreatectomy they were placed on the standard McCollum diet.

The operative technic has been described in a previous paper (5). In most instances we removed all of the pancreatic tissue except a small mass lying between the bile duct and the duodenum.

At the termination of the experiments we killed the rats, weighed the endocrine glands, and made a search for pancreatic tissue.

RESULTS

First Series

Carbohydrate, fat, casein, and yeast appetite of pancreatectomized rats. Figure 1B gives the yeast, casein, sucrose and olive oil intake of a rat which had been pancreatectomized at an age of 56 days and on the McCollum diet had constantly shown marked diabetic symptoms. It was started on the self-selection diet at an age of 98 days and was returned to the McCollum diet at the age of 134 days. Throughout the entire 36-day period on the self-selection diet, except for the first few days, this rat consistently avoided the sucrose and ate large amounts of olive oil, casein and yeast.

Five of the 7 rats gave similar records. During the first 3 to 10 days on the self-selection diet, they ate sucrose and little or no olive oil. After that they took olive oil and little or no sucrose and consistently made this choice throughout the rest of the experiment. One rat ate large amounts of olive oil for 5 days. During the next

TABLE 1. FIRST SERIES

Rat No.	No. of days on self-selection diet	Average daily intake for last 20 days on self-selection diet				Caloric intake		
		Olive oil	Sucrose	Casein	Yeast	Carbohydrate	Fat	Protein
		cc.	gm.	gm.	gm.	%	%	%
1	114	3.4	0.5	4.2	1.6	8	57	35
2	62	2.9	0.3	1.0	2.1	12	68	20
3	62	4.0	0.9	0.2	4.3	19	66	15
4	36	3.4	0.5	5.4	3.3	12	47	41
5	83	1.7	4.2	0.3	3.1	50	35	15
6	83	3.5	1.9	0.3	2.9	24	64	12
7	102	3.0	0.4	3.4	1.7	9	57	34
Average	106	3.1	1.2	2.1	2.7	19	56	25
Normals (8), Averages for last 10 days on self-selection diet								
Average	>40 Days	1.9	3.3	2.2	1.4	36	38	26

20-day period it averaged 22.7 gm. of sucrose per day and no olive oil, then changed back to olive oil again. One rat changed from one to the other at irregular intervals throughout the experiment.

Table 1 summarizes the results of the observations made on the 7 rats. The first 6 columns give the identification numbers of the rats, the number of days on the self-selection diet, and the average daily intake of the 4 substances for the last 20 days on the self-selection diet. At the bottom the table contains the averages for 8 normal rats of approximately the same age, which had been on the self-selection diet for at least 40 days. Six of the 7 pancreatectomized rats ingested large amounts of olive oil; one took only a moderate amount. Their olive oil intake averaged 3.1 cc., while the intake of the 8 normals averaged only 1.9 cc. Five rats ate almost no sucrose at all; one ate a small amount; one, a moderate amount. This latter rat is the one which showed the repeated changes in olive oil and sucrose appetite. The sucrose intake of the 7 rats averaged 1.2 gm., as compared to 3.3 gm. for the 8 normals. The casein intake failed to show any consistent changes. Three rats ate moderate to large amounts; 4 took very small amounts. Their intake averaged 2.1 gm., while the intake of the 8 normals averaged 2.2 gm. The yeast intake was about twice as high as the normal. It averaged 2.7 gm. for the pancreatectomized rats and 1.4 gm. for the controls.

Percentage of carbohydrate, fat, and protein intake. The last 3 columns of table 1 give the caloric intake in percentage of carbohydrate (sucrose and the carbohydrate in yeast), fat (olive oil and the fat in yeast), and protein (casein and the protein in yeast). Carbohydrate averaged 19% for the 7 rats and, with the exception of one rat which ate a large amount of sucrose, ranged from 8 to 24%. Fat intake averaged 56% and, with the exception of this same animal, ranged from 47 to 68%; casein averaged 25% and ranged from 12 to 41%. A comparison with the intake records for the 8 normals listed at the bottom of table 1 shows that the pancreatectomized rats took half as much carbohydrate, half again as much fat, and almost the same amount of protein as the normals.

Mineral appetite. None of the animals showed a change in appetite for sodium chloride or any of the other mineral solutions.

Effects of the dietary selections. Water intake. Figure 1A gives the water intake (tap water plus water from the mineral solutions) and body weight curves of the rat whose

TABLE 2. FIRST SERIES

Rat No. and sex	Age at:				Average daily water intake			Body weight at:			
	Pancreatectomy	Start of self-selection diet	Return to McCollum diet	Autopsy	Last 10–15 days on McCollum diet	Last 20 days on self-selection diet	Last 10 days on McCollum diet	Start of self-selection diet	End of self-selection diet	Autopsy	
	days	days	days	days	cc.	cc.	cc.	gm.	gm.	gm.	
1♂	27	164	278	335	70.1	15.3	104.2	170	235	224	
2♂	74	151	213	232	63.9	19.9	27.0	120	132	136	Died
3♂	62	110	172	186	98.0	25.3	27.0	110	181	167	Died
4♂	56	98	134	153	83.5	37.9	82.8	237	305	288	
5♀	60	209	292	318	52.2	34.5	73.2	170	166	160	
6♀	53	202	285	314	55.3	13.2	51.4	172	200	188	
7♂	40	202	304	358	82.0	28.2	29.3	206	245	268	
Average	53	162	240	271	72.1	24.9	56.4	169	209	204	

dietary selections were shown at the right. This rat, kept on the McCollum diet, was pancreatectomized at an age of 56 days, placed in the activity cages at 72 days, changed over to the self-selection diet at 98 days, and returned to the McCollum diet at 134 days. While on the McCollum diet it showed a marked polydipsia. The intake averaged from 50 to 60 cc. per day and on 2 days reached peaks of 120 and 122 cc. Almost immediately after it was placed on the self-selection diet the rat reduced its water intake to a lower level. After 30 days the intake averaged only about 40 cc. per day. When returned to the McCollum diet, the rat immediately increased its water intake again. After 15 days the intake reached a peak of 102 cc. Thus, the polydipsia, present on the McCollum diet, disappeared on the self-selection diet, and reappeared on the return to the McCollum diet.

Six of the rats gave essentially the same type of record, except that for the first few days immediately after the rats started on the self-selection diet, that is, while they still ate the sucrose, their water intake increased rather than decreased. Their water intake closely paralleled the sucrose intake. The rat which ingested such large amounts of sucrose for over 30 days manifested a maximal polydipsia. For this period its intake averaged over 180 cc. per day; on several days it drank more than 200 cc. When the rat stopped eating sucrose, its water intake dropped to a level near 35 cc. (fig. 5).

Table 2 summarizes the results of the observations made on the 7 rats. The first 8 columns give the identification numbers of the rats, their sex, their ages at pancreatectomy, at the start of the self-selection diet, at the return to the McCollum diet and at autopsy, and their average daily water intake for the last 10 to 15 days on the McCollum diet, for the last 20 days on the self-selection diet, and for the last 10 days

after their return to the McCollum diet. It also gives body weight at the start and end of the self-selection diet and at autopsy.

During the last 10 to 15 days before the rats started on the self-selection diet, their daily water intake averaged 72.1 cc., which is approximately 3 times the normal average for rats of this weight. On the self-selection diet the water intake of all 7 rats showed a definite decrease. For the last 20 days on the self-selection diet the water intake averaged only 24.9 cc. When returned to the McCollum diet, 4 of the 7 rats showed a return of the polydipsia. The average intake of one of the rats increased from 15.3 to 104.2 cc. per day. The 3 remaining rats drank only slightly more than they drank when on the self-selection diet (rats 2, 3, 7). Two rats died shortly after their return to the McCollum diet (rats 2, 3). At autopsy we found no pancreatic tissue in these rats. Both had had a second operation in which all of the small masses of pancreatic tissue around the bile duct were removed. These 2 rats must have been deprived of their external as well as of their internal secretion. At autopsy the other 5

TABLE 3. FIRST SERIES

Rat No.	Total calories per kg. body weight per day on:			
	Last 10 days on McCollum diet	Last 20 days on self-selection diet	Last 10 days on McCollum diet	
1	367	244	362	
2	—	287	260	Died
3	—	253	208	Died
4	—	213	278	
5	367	260	363	
6	340	272	315	
7	395	199	189	
Average	367	247	282	

10-Day average for normals (8) on self-selection diet, 245 (176–313 range of variation)

rats showed varying amounts of pancreatic tissue between the bile duct and the duodenum where it had purposely been left. There is a possibility that the rat which showed no change in water intake on the return to the McCollum diet may have had a sufficiently large amount of tissue to carry on an almost normal existence.

Body weight. Figure 1A gives a fairly typical body weight curve. When placed on the self-selection diet, this animal, which had been underweight, immediately began to gain weight at an increased rate for about 14 days. Then it continued to gain weight at the normal rate. When returned to the McCollum diet, the rat scarcely maintained its weight.

The last 3 columns in table 2 summarize the results of these observations. Six rats gained weight on the self-selection diet; one lost weight. The average increased from 169 gm. at the start of the diet to 209 gm. at the end. When returned to the McCollum diet, 5 rats lost weight; one showed no change; the other, a slight gain. This latter rat (7) also failed to show a polydipsia on the return to the McCollum diet.

Total caloric intake. On the McCollum diet the total caloric intake of the diabetic rats was definitely above the normal average; on the self-selection diet it decreased approximately to the normal level and increased again when the rats were returned to the McCollum diet. Table 3 gives the averages. The total caloric intake per kilogram of body weight was obtained on 4 of the rats on the McCollum diet before the start of the self-selection diet. It averaged 367 calories and ranged from 340 to 395. During the last 20 days on the self-selection diet the average decreased to 247 calories per

kilogram of body weight and ranged from 199 to 287. The 8 normal rats averaged 245 calories and ranged from 176 to 313. When the rats were returned to the McCollum diet, 4 of the 7 rats showed an increase in total caloric intake; 3 rats, a decrease. Two of these rats had lost all pancreatic tissue, had failed to show the polydipsia, and had died shortly after the return to the McCollum diet. The third rat apparently had sufficient pancreatic tissue to carry on normal metabolism.

Blood sugar. Determinations of blood sugar were made infrequently because the period of starvation and the necessary manipulation usually greatly disturbed the

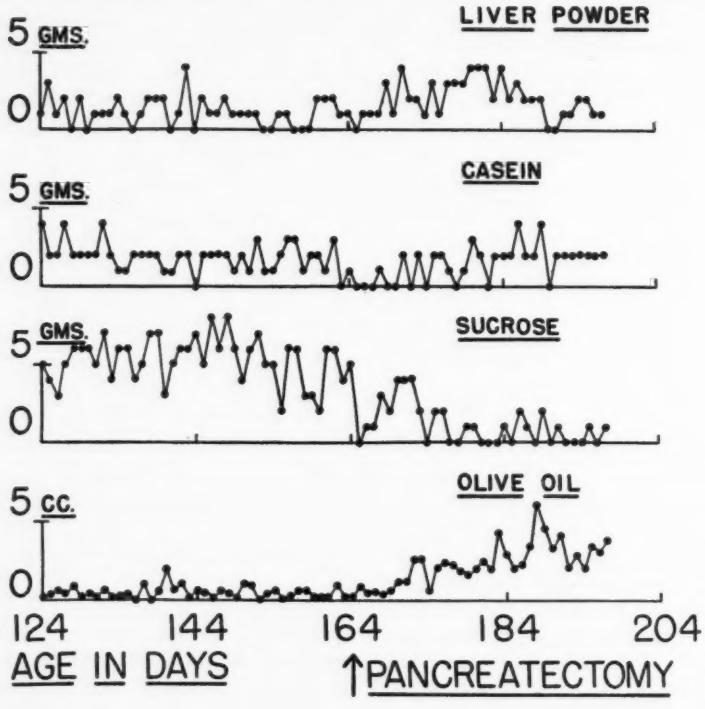

Fig. 2

other records. However, at least two determinations were made for each rat, one on the McCollum and one on the self-selection diet. Five to six determinations were made on some of the animals. For purposes of comparison, blood sugar readings were made on a group of normals of approximately the same age and kept on the McCollum diet. The blood was taken from the tail after a 15-hour fast and the determinations were made by the method of Folin and Malmros (6). The blood sugar readings on the 7 pancreatectomized rats on the McCollum diet just before the start of the self-selection diet averaged 286 mg. per 100 cc. and ranged from 160 to 355 mg. %. Readings on the normals averaged 95 mg. %. On the self-selection diet, not always near the end of the period of this diet, the readings averaged 138 mg. % and ranged from 93 to 199 mg. %. Urine sugar determinations were made with the Benedict qualitative solution. The usual plus 3 and 4 reactions present while the animals received the McCollum diet changed to negative, or at most plus 1, when the rats had been on the self-selection diet for some time.

Spontaneous activity. On the McCollum diet the diabetic rats were almost totally inactive. When placed on the self-selection diet, 2 of the rats showed a moderate activity; the other 5 remained very inactive. One of the 2 moderately active rats still showed some activity when returned to the McCollum diet.

These results agree with our previous experiments which showed that a fairly complete removal of the pancreas made rats almost totally inactive. Very small unremoved pieces of pancreatic tissue sufficed for maintenance of a moderate amount of activity. It is noteworthy that the 2 rats, 5 and 6, which showed a moderate amount of activity, also showed the smallest diabetic effects. These were the only rats which ate any sugar. It is very likely that they retained larger amounts of functioning islet tissues than any of the other rats.

These experiments did not indicate that the dietary selections made by the rats returned their activity to a normal level. It must be remembered, however, that several months elapsed between the pancreatectomy and the start of the self-selection diet, and that during this interval the rats probably had been extremely inactive. In other experiments we have found that rats which have been inactive for a long time can be restored to normal activity only with great difficulty.

TABLE 4. SECOND SERIES. INTAKE OF DIFFERENT FOODSTUFFS

Rat No.	Sucrose		Olive oil		Casein		Yeast		Liver	
	Last 10 days before operation	Last 10 days on self-selection diet	Last 10 days before operation	Last 10 days on self-selection diet	Last 10 days before operation	Last 10 days on self-selection diet	Last 10 days before operation	Last 10 days on self-selection diet	Last 10 days before operation	Last 10 days on self-selection diet
	gm.	gm.	cc.	cc.	gm.	gm.	gm.	gm.	gm.	gm.
8	4.3	0.5	0.4	3.2	1.8	2.0			0.9	1.2
9	0.4	0.5	1.9	2.6	2.2	1.0			1.1	0.6
10	3.8	0.2	1.5	4.6	1.5	0.6			1.5	0.8
11	2.3	1.6	1.7	2.7	4.3	3.3			1.2	2.1
12	0.6	0.4	4.0	4.7	3.1	3.4	0.7	1.3		
13	3.2	0.1	1.8	4.8	2.8	2.8	1.3	1.1		
14	9.8	0.4	0.1	3.1	0.4	3.6	2.5	2.6		
15	1.8	0.7	3.7	4.3	1.6	1.6	1.0	2.1		
Average	3.3	0.6	1.9	3.7	2.2	2.3	1.4	1.8	1.2	1.2

Second Series

Fat, carbohydrate, protein, and yeast or liver powder appetite of pancreatectomized rats. Figure 2 gives the liver powder, casein, sucrose and olive oil intake of a rat for 40 days before pancreatectomy and 33 days afterwards. The pancreas was removed when the rat was 164 days of age. It was returned to the McCollum diet when it was 197 days of age. Before pancreatectomy it showed very little appetite for olive oil. Its daily intake averaged less than 0.5 cc. Within a few days after pancreatectomy it began to take the olive oil; and after 20 days, its daily intake reached a level over 3 cc. On the first postoperative day the rat ate no sucrose at all, but then for a few days ate it again, however, only in small amounts. After 7 days it refused it almost altogether. After a temporary decrease which lasted about 10 days, the casein intake returned to its preoperative level. Within 5 days after pancreatectomy the liver powder intake increased to a higher level at which it remained fairly constantly to the end of the experiment.

Table 4 summarizes the results. Six of the 8 rats ate sucrose before pancreatectomy. After pancreatectomy 5 of these 6 rats ate almost no sucrose; 1 rat still ate a small amount. Two rats, which before pancreatectomy ate almost no sucrose, still continued to avoid the sucrose. The average daily intake for the 8 rats decreased from 3.3 gm. for the last 10 days before operation to 0.6 gm. for the last 10 days on the self-

selection diet. All 8 rats increased their olive oil intake after pancreatectomy. The 10-day average increased from 1.9 to 3.7 cc. The casein intake decreased in 3 of the rats, increased or remained unchanged in the other 5 rats. The yeast and liver powder intake increased in 5 rats and decreased in the other 3 rats.

Percentage intake of carbohydrate, fat and protein. Table 5 gives the percentage caloric intake of carbohydrate, fat and protein for the last 10 days on self-selection before pancreatectomy and for the last 10 days on the self-selection diet after pancreatectomy. Before pancreatectomy carbohydrate constituted 36% of the diet, afterward only 9%; fat intake increased from 38 to 68%; protein intake remained practically unchanged. The fat intake increased in all 8 rats. After pancreatectomy the carbohydrate intake ranged from 4 to 19%; the fat intake, from 48 to 88%.

Effects of dietary selections. Water intake. Figure 3 gives the water intake and body weight curves of the rat whose dietary selections were presented in figure 2. This rat

TABLE 5. SECOND SERIES

Rat No.	Caloric intake					
	Last 10 days on self-selection before operation			Last 10 days on self-selection after operation		
	Carbohydrate	Fat	Protein	Carbohydrate	Fat	Protein
	%	%	%	%	%	%
8	60	12	28	9	67	24
9	11	55	34	10	74	16
10	44	34	22	4	88	8
11	24	34	42	19	48	33
12	8	68	24	5	71	24
13	34	36	30	4	74	22
14	86	2	12	11	53	36
15	18	66	16	11	71	18
Average	36	38	26	9	68	23

remained on the self-selection diet for 73 days, 40 days before pancreatectomy and 33 days afterwards. On the basis of the water intake record on the self-selection diet, it would appear that the pancreatectomy had had no effect. However, after the return to the McCollum diet, the sharp increase to a very high level (139 cc. on the last day before the rat was killed) demonstrates that the pancreatectomy had produced a marked effect. On the multiple choice diet the selections prevented the appearance of the polydipsia.

Table 6 summarizes the results. The daily water intake for the 8 rats averaged 28.7 cc. for the last 10 days before pancreatectomy and only 28.5 cc. for the last 10 days before they were changed to the McCollum diet. That the pancreatectomy had a marked effect is shown by the sharp increase in water intake which occurred when the rats were placed on the McCollum diet. In 5 of the rats the water intake increased; in 2 it decreased slightly. In 1 rat the water intake increased from a daily average of 27.3 cc. to 89.3 cc. One rat was killed at the end of the self-selection period. At autopsy small masses of pancreatic tissue were found in all of the rats between the bile duct and the duodenum.

Body weight. Figure 3 shows that in this rat, kept on the self-selection diet, pancreatectomy produced only a slight decrease in body weight. Twenty days after pancreatectomy the animal had not only regained its preoperative weight but showed a small additional gain. After the return to the McCollum diet, the animal began to lose weight.

Table 6 summarizes the results. While on the self-selection diet, 7 of the 8 rats gained weight; the other one showed no change. The average for the 8 rats increased from 180 to 197 gm. At autopsy, performed after their return to the McCollum diet, the weights tended to remain the same. The rats neither lost nor gained very much.

Total caloric intake on the self-selection and McCollum diets. Table 7 summarizes the results. The total calories per kilogram of body weight did not change appreciably

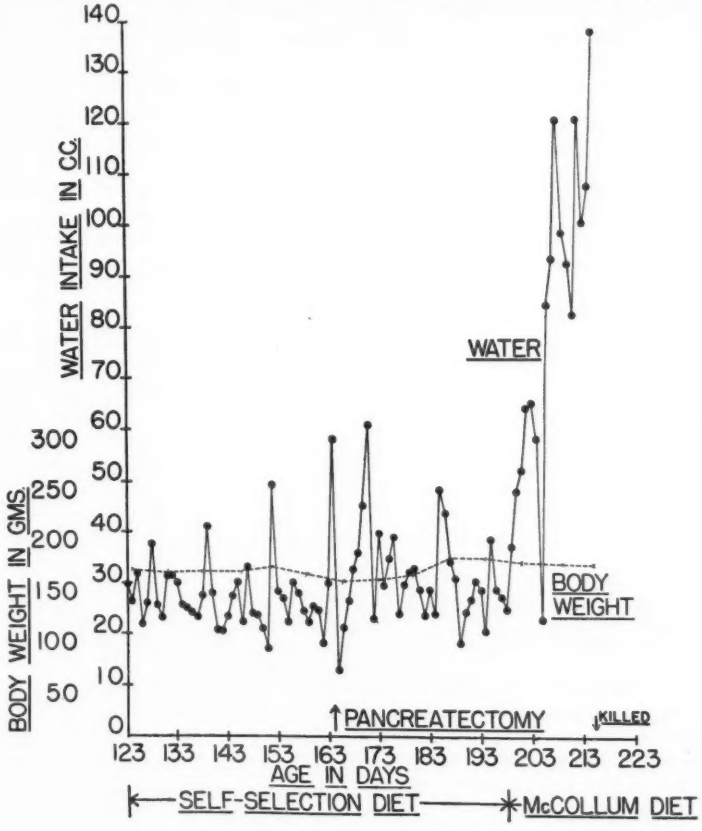

Fig. 3

after pancreatectomy while the rats remained on the self-selection diet. It averaged 245 calories for the last 10 days before pancreatectomy and 256 calories for the last 10 days on the diet. On the McCollum diet it increased markedly in 5 of the 7 rats on which records were obtained; it decreased slightly in 2 rats. For the 7 rats it averaged 342 calories. This agrees closely with the caloric intake average of 367 for the pancreatectomized rats of the first series before they were placed on the self-selection diet.

Blood sugar. In the second series we did not make blood sugar determinations.

Spontaneous activity. Table 8 summarizes the results of these experiments. Five of 8 rats became less active after pancreatectomy; 3 became more active. The activity level of 7 of the rats after pancreatectomy was definitely much higher than that of the

TABLE 6. SECOND SERIES

Rat No. and sex	Age at:			Water intake			Body weight at:		
	Pancreatectomy	End of self-selection diet	Autopsy	Last 10 days before pancreatectomy	Last 10 days on self-selection diet	First 20 days on McCollum diet	Pancreatectomy	End of self-selection diet	Autopsy
	days	days	days	cc.	cc.	cc.	gm.	gm.	gm.
8 ♀	164	197	216	23.7	28.5	61.4	153	172	170
9 ♀	164	197	218	26.6	20.1	59.1	175	187	185
10 ♀	136	169	187	32.8	20.9	17.5	182	195	194
11 ♀	173	199	218	31.4	29.7	14.5	231	231	238
12 ♀	107	259	259	26.5	37.8	—	185	200	200
13 ♀	104	143	255	27.2	27.3	89.3	164	193	193
14 ♀	100	139	251	38.8	36.3	62.2	180	205	208
15 ♀	100	139	251	22.2	27.0	54.2	170	195	214
Average	131	180	232	28.7	28.5	51.2	180	197	200

previously reported rats kept on the McCollum diet (5). After the return to the McCollum diet, 3 of the rats became less active; 4 became more active.

These results indicate that the dietary selections made by the rats also had an effect on spontaneous activity. In order to explain the failure of all of the rats to become

TABLE 7. SECOND SERIES

Rat No.	Total calories per kg. body weight per day		
	Last 10 days before pancreatectomy	Last 10 days on self-selection diet	Last 10 days on McCollum diet
8	206	252	476
9	176	163	356
10	176	206	294
11	265	280	245
12	313	308	—
13	273	304	426
14	280	256	354
15	272	276	246
Average	245	256	342

inactive on the return to the McCollum diet, we may think first of the possibility that large remnants of pancreatic tissue remained intact. The appearance in most instances of definite symptoms of diabetes indicates, however, that not much tissue could have remained. It is possible that on the high fat diet the remaining islet cells had an opportunity to recuperate sufficiently to maintain the rats at a fair level of activity even on the McCollum diet (7).

TABLE 8. SECOND SERIES. SPONTANEOUS RUNNING ACTIVITY

Rat No.	Self-selection diet		McCollum diet
	Last 10 days before pancreatectomy	Highest 10-day average after pancreatectomy	Highest 10-day average
8	8,689	1,289	942
9	9,032	658	939
10	8,338	9,416	10,936
11	6,810	951	7,164
12	4,335	7,005	—
13	12,489	7,358	2,417
14	11,359	3,586	4,135
15	12,580	16,040	8,627

Further observations on the increased yeast appetite. The results indicate that the pancreatectomized rats may have an increased appetite for yeast. Since yeast contains so many different substances, members of the vitamin B complex—thiamin, riboflavin, nicotinic acid, B_6—in addition to the carbohydrate, fat and protein, it is difficult to determine the basis for any increases in appetite for yeast. We do know that vitamin B deficient rats have a markedly increased appetite for yeast, and apparently for the vitamin B content (8). In the present experiments the rats had access to carbohydrates fats, and proteins, separate from that in the yeast. It would seem likely, therefore, that the rats did not take the yeast to get additional amounts of any of these substances. We do not know whether the rats sought one, several, or all of the vitamin

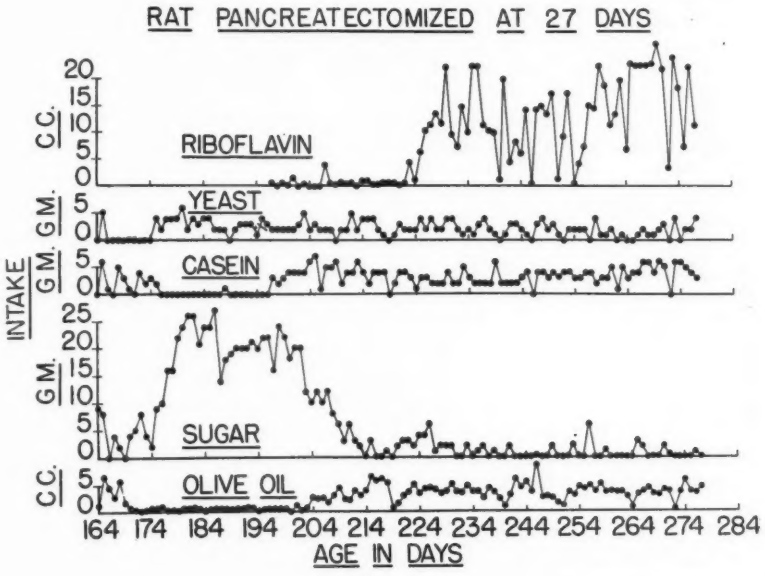

Fig. 4

B components. Further experiments in which rats have access to the components in separate form rather than combined as in yeast may reveal the desired substance.

One of the rats used in the first series had access to a solution of crystalline riboflavin in a separate container for part of the time that it was on the self-selection diet. The results of these observations, however inconclusive, seem sufficiently suggestive to warrant a more detailed account. Figure 4 contains a record of the dietary selections made by this rat that was pancreatectomized at the age of 27 days and started on the self-selection diet at the age of 164 days. After about 10 days it started to eat yeast and large amounts of sucrose, eating as much as 25 gm. on several days, and stopped taking olive oil and casein. On the 32nd day of this diet, when the rat was 196 days old and the sucrose intake averaged about 20 gm., riboflavin in a 0.01% solution was made available in a graduated inverted bottle. The rat took a very small amount of the solution each day. Of interest is the fact that on the next day the rat at once started to eat casein again in normal amounts; in 6 days the sucrose intake began to decrease, and the olive oil intake increased. Twenty days after the riboflavin was offered, the rat had stopped eating sucrose almost entirely and ingested large amounts

of olive oil. Its intake levels of these 2 substances remained unchanged throughout the rest of the experiment. After the riboflavin was made available, the rat started to drink much smaller amounts of water and 40 days later its water intake averaged near 35 cc. per day. Figure 5 gives the water intake and body weight curves of this rat. During the time that the rat ate such large amounts of sucrose, it drank very large amounts of water, as much as 239 cc. on one day; and it failed to gain weight. Almost immediately after it started on the riboflavin it began to gain weight. Its body weight increased from a low point, 172 gm., to 240 gm. When returned to the McCollum diet, the water intake increased again to a level near 80 cc. per day; and it failed to maintain its body weight. Although the results do not permit any conclusions to be

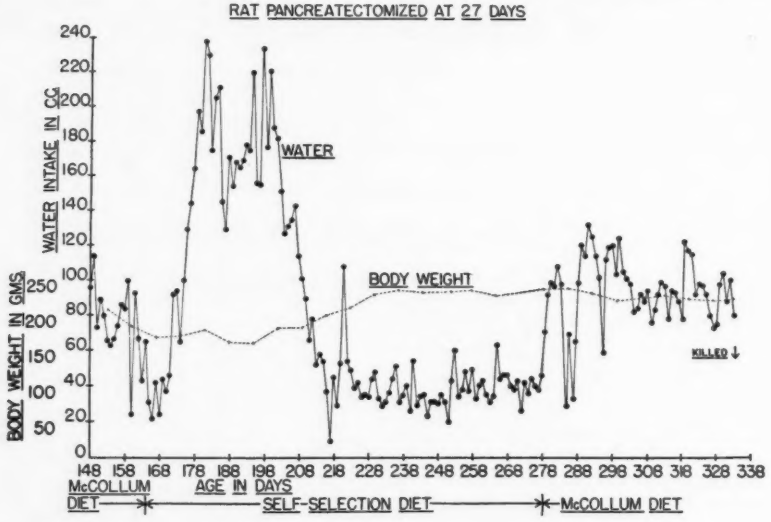

Fig. 5

drawn, it would appear that the ingestion of riboflavin may have helped the rat to use the protein casein, and in some way indirectly to overcome the diabetic symptoms. In keeping with such an interpretation we have reported that riboflavin plays a part in the stimulation of the appetite for protein (4).

DISCUSSION

These experiments furnish further evidence for the operation of behavior factors in the regulation of the internal environment. On the basis of the results of these experiments we have attempted to formulate the mechanisms involved in production of experimental diabetes in the rat. In the first place, as is well known, removal of the pancreas abolishes the ability of animals to utilize and store carbohydrate with the result that sugar accumulates in the blood in large amounts. Sugar excreted from the kidneys carries with it water, causing a flow of fluid from the tissues to the blood stream. In response to the resulting dehydration the animals seek and ingest large amounts of water. Here, just as in diabetes insipidus, the polydipsia follows the polyuria. On the McCollum diet, in which the rats were forced to take carbohydrate, fat, and protein in fixed proportions, a large part of the ingested energy constituted by the carbohydrate must have passed through the body unused. This must have given rise

to hunger, which the rats attempted to satisfy by eating more food. However, as the food intake increased, the carbohydrate also increased; and as a consequence, the diabetic symptoms became further intensified. The animals had to strike a balance between the desire to satisfy their hunger and an attempt to maintain a normal sugar balance of the blood.

When placed on the self-selection diet, the rats were no longer forced to take carbohydrate, except for the small amount contained in the yeast. They stopped eating sucrose and ate large amounts of olive oil; and as a consequence, the water was no longer drained from the tissues. The thirst disappeared, energy from the sugar was no longer lost; so the food intake decreased to its normal level again. With the return to the McCollum diet, the symptoms were reversed again.

The dietary selections made by the diabetic rats closely agree with the diets determined empirically by clinicians from human diabetics in the preinsulin period. It was generally agreed that patients did much better on a high fat than on a low fat diet. Since insulin increases the ability to utilize carbohydrate, the need for a high fat diet is no longer present. In preliminary experiments we have found that, when treated with insulin, our diabetic rats stop taking olive oil in such large amounts and eat more sucrose.

These results also agree with the recent experiments of Best, Haist, and Ridout and of Haist and Best (7, 9), who found that the insulin content of the islets of rats kept on a high fat diet is much lower than in rats kept on regular diets. According to them, this demonstrates that fat "rests" the islet cells of Langerhans. It follows that rats on a high fat diet have smaller need for the islet secretions.

The increased yeast or liver powder appetite manifested by most of the rats probably indicates an increased desire for some of the components of the vitamin B complex. We know that the rats did not eat the yeast for the carbohydrates; and since protein was available in the form of casein, it is likely that they did not eat it to get more protein. We reported a few observations which indicated that they might want riboflavin. Definite information regarding this point can be obtained by experiments in which the yeast is replaced by crystalline thiamin, riboflavin, nicotinic acid and B_6, offered in separate containers in addition to the other ingredients of the self-selection diet. We know that, when given access to this assortment of substances, normal rats show good growth and development. Thus, it should be possible to use these same substances with diabetic rats.

In all of the self-selection experiments made so far we have found an inverse relationship between the appetite for olive oil and sucrose. We have never found that rats take large amounts of both. When they increase one, they invariably decrease the other.

These experiments do not permit any definite conclusions to be drawn regarding the dependence of spontaneous activity of the pancreatectomized rats on the fat and carbohydrate content of the diet. In agreement with results of a previous experiment it was found in the first series that rats pancreatectomized on the McCollum diet were extremely inactive. The inactivity we attempted to explain in terms of their inability to utilize carbohydrate. The fact that the pancreatectomized rats on a high fat diet remained inactive would be in accord with these previous findings. It is possible that, had the rats of the second series been more completely pancreatectomized, they too would have become inactive even on the self-selection diet, because of the inability to utilize carbohydrate.

Clinically it was long held that diabetic patients should refrain from exercise. Since the introduction of insulin treatment and with it the partial reestablishment of the ability to utilize carbohydrate, exercise has not only been recommended but has

been found to be beneficial. It will be interesting to know whether insulin treated pancreatectomized rats will become normally active again.

SUMMARY

Seven early pancreatectomized rats, which on the standard McCollum diet for at least several weeks had shown marked diabetic symptoms—polydipsia, increased food intake, decreased rate of growth and hyperglycemia—were placed on the self-selection diet for a month or more, and then were returned to the McCollum diet. On the self-selection diet they had access to 11 purified (or nearly purified) substances in separate containers.

Six of the 7 rats manifested a marked appetite for the fat, olive oil, and ate little or none of the carbohydrate, sucrose, All 7 rats had an increased appetite for yeast. Three rats ate large amounts of casein. In this diet the intake of carbohydrate averaged 19%; fat, 56%; and protein, 25%.

The dietary selections made by the rats had pronounced effects on their diabetic symptoms. The polydipsia, the increased appetite, and the hyperglycemia either disappeared or were greatly reduced; and the rats gained weight almost at the normal rate. When returned to the McCollum diet, 4 of the 7 rats showed diabetic symptoms again.

Eight rats were kept on the self-selection diet for at least 40 days before they were pancreatectomized and for an equal period afterwards. Then they were put on the McCollum diet. After pancreatectomy all of the rats ate more fat and little or no carbohydrate. Five ate more yeast or liver powder; 3 ate less. Three ate more protein; 3 ate less and 2 showed no change. As measured in calories, the intake of carbohydrate changed from 36 to 9%; fat, from 38 to 68%; and protein remained unchanged, 26 to 23%.

So long as these pancreatectomized rats remained on the self-selection diet and were thus able to regulate their own diet, diabetic symptoms did not appear. However when they were placed on the McCollum diet, 5 of the 8 rats showed a polydipsia, increased food intake, reduced rate of gain of weight and hyperglycemia.

Thus, by their dietary selections the rats helped to correct the diabetic symptoms. Their selections agree closely with our knowledge of the needs of human diabetics. Before the introduction of the insulin treatment it had long been established that diabetics thrive better on a high fat diet, that they are unable to use carbohydrate.

It was shown that the various responses of the diabetic rats can be explained in terms of Claude Bernard's principle of the maintenance of the internal environment.

These experiments were supported in part by a grant from the Committee on Research in Endocrinology of the National Research Council for the study of appetite and the internal secretions. We are also indebted to Merck & Co., Inc. for their generous supply of riboflavin, to the Northwestern Yeast Co. for the yeast powder, and to Mead Johnson & Co. for the liver powder.

REFERENCES

1. Richter, C. P.: *Am. J. Physiol.* 115: 155. 1936.
2. Richter, C. P., and J. F. Eckert: *Am. J. Med. Sc.* 198: 9. 1939.
3. Richter, C. P., and B. Barelare, Jr.: *Endocrinology* 23: 15. 1938.
4. Richter, C. P., and B. Barelare, Jr.: *Am. J. Physiol.* 127: 199. 1939.
5. Richter, C. P., and E. C. H. Schmidt, Jr.: *Endocrinology* 25: 698. 1939.
6. Folin, O., and H. Malmros: *J. Biol. Chem.* 83: 115. 1929.
7. Haist, R. E., and C. H. Best: *Science* 91: 410. 1940.
8. Richter, C. P., L. E. Holt, Jr., and B. Barelare, Jr.: *Am. J. Physiol.* 122: 734. 1938.
9. Best, C. H., R. E. Haist and J. H. Ridout: *J. Physiol.* 97: 107. 1939.

BEHAVIOR AND ENDOCRINE REGULATORS OF THE INTERNAL ENVIRONMENT

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THE RESULTS of experimental studies made during the last 20 years in this and other laboratories on the spontaneous activity of animals, particularly of the common laboratory rat, have demonstrated the ability of animals to regulate their food and water intake to their internal needs under a great variety of normal and pathological conditions (1–4). Recently we have found that rats with various endocrine deficiencies can make dietary selections which compensate for their deficiencies. Until about 3 years ago such ability remained an interesting, isolated behavior phenomenon. But it is realized now that this ability can be understood in terms of a broad biological concept first stated in 1859 (5) by the great French physiologist, Claude Bernard—the concept of constant internal environment. Bernard distinguished an external environment, which surrounds all objects and with which we are all familiar, from an internal environment comprised of the fluids of the body which form the optimal habitat for living cells. He stated, "It is the fixity of the 'milieu interne' which is the condition of free and independent life; and all the vital mechanisms, however varied they may be, have only one object, that of preserving constant the condition of life in the internal environment." Cannon (6) greatly elaborated and extended this concept and spoke of a steady state or homeostasis. He listed the water, salt, calcium, sugar and fat content of the blood, as well as oxygen, acid-base balance, and body temperature as some of the factors which must be maintained within exceedingly narrow limits if the body is to persist in a state of health.

Bernard and Cannon emphasized some of the individual factors which help to maintain a constant internal environment, such as, for instance, increased rate and depth of respiration, sweating, burning of fat, etc. In addition, we believe that the ability of animals to maintain a constant internal environment, after certain single organs or physiological regulators have been removed, clearly establishes the existence of behavior or total organism regulators. A few examples, taken partly from previous papers and partly from unpublished data, will suffice to show what is meant by the term, behavior regulators.

The first example deals with the maintenance of an internal water balance. The posterior lobe of the pituitary serves as one of the chief physiological regulators of water metabolism. Removal of the posterior lobe eliminates the antidiuretic hormone with the result that the organism excretes urine in large amounts and, if given only the normal amount of water, quickly becomes dehydrated and dies. If, however, the animals have access to an unlimited supply of water, they will drink sufficiently large amounts to keep themselves alive and in good health (7). This condition, characterized by polyuria and polydipsia, is known as diabetes insipidus. The increased water intake represents to us an effort of the animal to maintain a constant internal environment, compensating for the polyuria, which is primary.

Received for publication August 20, 1940.

The second example concerns the maintenance of a constant body temperature. The anterior lobe of the pituitary and the thyroid constitute the chief physiological regulators of body temperature. Removal of either of these glands results in decreased heat production and a threatened drop in body temperature. When animals, deprived of these glands, remain in even slightly lowered external temperatures, they die within a few days. We have found that, given the opportunity, such animals will make an effort to maintain a constant body temperature by building large nests and hiding themselves in them, thus at least partially compensating for the decreased heat production by an increased heat conservation. The cages used for these observations have been previously described (8). The end of a roll of soft tissue paper hangs freely in the inside of the cage within easy reach of the rat, and cyclometers record the length of paper strips pulled off the roll. All nest paper is removed daily, thus necessitating the construction of a new nest each day. Under constant conditions of temperature the amount of paper used daily remained very nearly constant and was approximately the same for both sexes. Cold external temperatures greatly increased nest building activity; warm temperatures reduced it. Removal of the thyroid gland or of the anterior lobe of the pituitary gland almost at once markedly increased nest building to a permanently high level. Some of these rats used 500 feet of paper each day. Thus, after thyroidectomy or anterior lobectomy and the resultant decrease in heat production had threatened the maintenance of a constant body temperature, the rats themselves made an effort to maintain a constant body temperature by building large nests around their bodies, thereby reducing heat loss.

The third example concerns the maintenance of a constant internal environment of electrolytes, fats, carbohydrates and proteins. It has been known for some time that removal of the adrenal glands greatly increases the loss of sodium in the urine with the result that the animal develops symptoms of insufficiency and dies within a few days. We have shown that when they are given access to sodium chloride solution in a container separate from the food adrenalectomized rats ingest sufficiently large amounts (from 5 to 20 times the amounts taken by the same animals before operation) to keep themselves alive and free from symptoms of insufficiency (9). Likewise, we know that removal of the parathyroids reduces the blood calcium with the result that animals develop tetany and often die. Given access to calcium solutions, they will ingest sufficiently large amounts to keep themselves alive and free of convulsions. Some rats drank 20 times as much calcium lactate solution after parathyroidectomy as before operation (10).

Further, it was found that after pancreatectomy the rats make dietary selections which eliminate their diabetic symptoms. In one series of experiments we used pancreatectomized rats which on the stock diet had shown marked symptoms of diabetes for several months—high blood sugar, high food intake, polydipsia and loss of weight. When placed on the self-selection diet, they chose large amounts of fat, little or no carbohydrate, and large amounts of yeast or liver powder, both of which are rich in the vitamin B complex. When returned to the regular mixed diet, all of the diabetic symptoms appeared again (Richter and Schmidt, in press).

In another series we pancreatectomized rats which had been on the self selection diet for several months. They, too, immediately started to eat more fat, stopped eating carbohydrate, ate more yeast and liver powder, and failed to develop diabetic symptoms. When placed on the regular stock diet, marked diabetic symptoms appeared—hyperglycemia, high polydipsia, increased food intake, loss of weight.

Numerous instances of self-regulation in human beings have also been observed. After destruction of the posterior lobe, human beings, like the diabetic rats, drink large amounts of water. We have found that patients suffering from destruction of the adrenal cortex will have an increased appetite, amounting to an actual craving in

many instances, for one or another of substances which have a high salt content—salty ham, sauerkraut juice, salted tomato juice, soups, etc. Dr. Lawson Wilkins and I recently reported the case of a 3-year-old boy who from the age of one year had eaten large amounts of salt, literally by the handfuls as other children like to eat sugar. Placed on a routine hospital diet with the normal amount of salt, he died within a week. Autopsy showed that a tumorous growth had destroyed the cortex of both adrenal glands (11). Dr. George Thorn has a patient with Addison's disease on his ward at the present time. This patient eats salt in very large quantities, literally covering his food with it. In addition, he drinks large amounts of heavily salted tomato juice, also salting his oranges, grapefruit and even lemons. He dislike sweets. Instances of increased calcium appetite in patients with a parathyroid deficiency have also been recorded; and it seems probable that this is the true explanation of plaster eating.

It is unfortunate that parental influences, advertisements and food fads tend to confuse the dietary selections made by human beings. Children are taught not to trust their appetites but to follow often faulty parental guidance. Defects in the gustatory apparatus or in the taste areas of the brain may also confuse selections. Taste threshold tests made on over 300 apparently normal individuals revealed many instances of taste blindness, even to such substances as sugar.

There can be very little doubt that in normal human beings, as well as in animals, both factors, the isolated organ and the total organism factors, play important parts in the maintenance of a constant internal environment. The relative importance of the two factors we do not know. It is likely that when one becomes weakened the other takes over more of the regulation. Thus, individuals with impaired adrenals may constantly eat more salt, while the adrenal cortex may become more active in individuals deprived of the normal salt intake.

Finally, we would like to point out that the self selection activities of animals may serve as an instrument for the determination of the function of the various endocrine glands. Thus, for instance, long before the develpoment of modern biochemical methods, we could have discovered the sodium function of the adrenals simply by putting the question to the rats. Given access to a variety of electrolytes, the rats would have quickly told us by their selections that adrenalectomized animals need sodium, similarly, that parathyroidectomized rats need calcium.

In conclusion, it has been shown that in interpreting endocrine studies, whether on human beings or on animals, we must take into account the fact that the organism itself may take an active part in compensating for deficiencies. Further, it has been pointed out that the total organism regulation, like the isolated organ regulation, can be understood in terms of Claude Bernard's concept of the maintenance of a constant internal environment.

This work was supported by grants from the Rockefeller Foundation and the Committee on Research in Endocrinology of the National Research Council.

REFERENCES

1. Richter, C. P.: *Comp. Psychol.* Mon. 1: 1. 1922.
2. Richter, C. P.: *J. Exper. Zool.* 44: 397. 1926.
3. Richter, C. P.: *Quart. Rev. Biol.* 2: 307. 1927.
4. Wang, G. H.: *Am. J. Physiol.* 71: 729. 1925.
5. Bernard, C.: Lecons sur les proprietes physiologiques et les alterations pathologiques des liquides de l'organisme. Paris, Bailliere, 1859.
6. Cannon, W. B.: The Wisdom of the Body. New York, Norton & Company, Inc., 1932.
7. Richter, C. P.: *Proc. Assoc. Res. Nerv. & Ment. Dis.* 17: 392. 1936.
8. Richter, C. P.: *Cold Spring Harbor Symp. on Quant. Biol.* 5: 258. 1937.
9. Richter, C. P.: *Am. J. Physiol.* 115: 155. 1936.
10. Richter, C. P., and J. F. Eckert: *Am. J. Med. Sc.* 198: 9. 1939.
11. Wilkins, L., and C. P. Richter: *J.A.M.A.* 114: 866. 1940.

AN ASSAY METHOD FOR HUMAN CHORIONIC GONADOTROPIN

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Since the discovery of chorionic gonadotropic substance, a great variety of units and assay methods have been used by different workers for quantitative determinations. The existence of so many methods is evidence in itself that none has been particularly satisfactory. The present work, previously published in abstract (1), was undertaken with the aim of devising an assay method which would have quantitative accuracy based upon objective data, and yet would eliminate the need for microscopic sections, i.e., a method which would be accurate and rapidly carried out, and simple enough to be used freely without prohibitive numbers of animals and excessive technical work. The increase in uterine size and fluid content as a secondary effect in immature rats and mice treated with gonadotropic substance has been observed by many workers (2–13). Levin and Tyndale (14), have used the uterine weight response in the immature mouse as a satisfactory assay for the gonadotropic substance from castrate urine, and Heller, Lauson and Sevringhaus (15) have used immature rats for pituitary gonadotropic material. Hamburger and Pedersen-Bjergaard (16) found in the rat, that the uterine and vaginal responses to chorionic gonadotropin were more reliable as end-points than were ovarian changes. D'Amour and D'Amour (17), in recently published work on the International Standard chorionic gonadotropin, have reached the same conclusions. Sealy and Sondern (18) have suggested a combination of uterine and ovarian weights for assay of this material over a wide range. These three groups of workers have used a 96 to 100-hour test period.

In beginning this work, a number of preliminary assays in immature rats, using chorionic gonadotropin obtained from the serum of pregnant women, and increasing the test period at 12-hour intervals from 36 hours to 96 hours, showed the maximum uterine weight increase at 72 hours. There was available at that time no material for construction of a standard curve to which assay results might be referred. Various commercial preparations of pregnancy urine gonadotropin varied so widely from one lot to another, that no reproducible standard for comparison could be expected. A preparation of chorionic gonadotropin was then made from pooled serum of pregnant women, and kept as a dry powder. It retained its original potency and the results reported here are based upon this material.

METHODS

The chorionic gonadotropin was prepared by pooling equal amounts of serum from 100 normal pregnant women in the last month of gestation. This was treated by a modification of the method of Fluhmann (7), to assure complete removal of estrogens. The serum was extracted 5 times with ether, precipitated with 6 volumes of 95% alcohol and the precipitate washed with ether. The powdered material was then

Received for publication June 27, 1940.
[1] Fellow, E. R. Squibb and Sons.

extracted with acetate buffer solution at pH 4.4, reprecipitated with alcohol and washed with ether. This yielded a water-soluble powder with an equivalent of 6.56 mg. per cc. of original serum. The preparation was tested for estrogenic activity and found to have none when given in 5 mg. doses. For clinical assays it was found unnecessary to carry out the second extraction and reprecipitation; a fine suspension of the crude first precipitate can be made and used directly. Such crude preparations showed no estrogenic activity when assayed in equivalent amounts.

Graded doses of this pooled serum gonadotropin were given to immature rats (hooded strain) selected for age (21 to 23 days) and weight (34 to 42 gm.). Age was

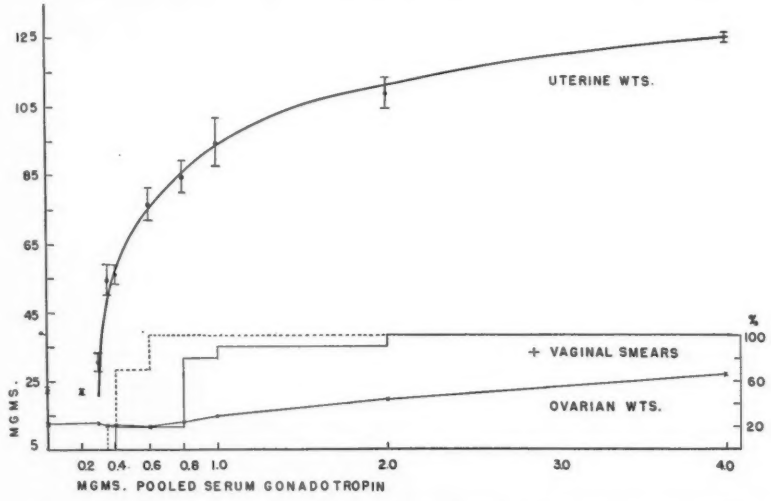

Fig. 1. The 72-hour response of uterine weight, ovarian weight and vaginal changes to graded doses of pooled serum gonadotropin. The plotted weight is the mean of all of the animals at each dosage level. The brackets show the standard error of the mean. Vaginal response is represented as percentage of animals showing full cornified response (solid line) and microscopic and mucoid response (broken line).

the more important, as rats older than 23 days formed corpora more readily and the uterine weight response was less reliable. The total dosage of gonadotropin was given in 6 divided subcutaneous injections, 3 injections being given daily for 2 days. Seventy-two hours after the first injection the animals were autopsied. Several groups of animals with the same dosage were autopsied at 96 hours, for comparison. The uteri were freed of mesometrium and severed at the tubo-uterine junction, and at the junction between body of the uterus and cervix. In this series of assays all of the uteri were weighed both with and without the cervices, the former weights being consistently 10% higher. Since the junction between body and cervix is sharply demarcated and no dissection is required, this has been adopted as the preferable method, and the weights given on the charts are of uterus without cervix. Correction of uterine weight for body weight did not make any significant difference in results, hence this correction has not been used. In order to check other criteria against the uterine weights, the ovaries were dissected free, weighed, examined grossly and microscopically. Vaginal smears were made and when a full cornified response was not present, sections were taken.

RESULTS

The observed mean uterine weights in milligrams when plotted directly against the dosage of serum gonadotropin in milligrams approach a curve as shown in figure 1 of the equation

$$Y = 98.63 + 44.28 \log (X - .27)$$

where Y is the response and X the dose. On semilog paper the relationship appears as a straight line (fig. 2).

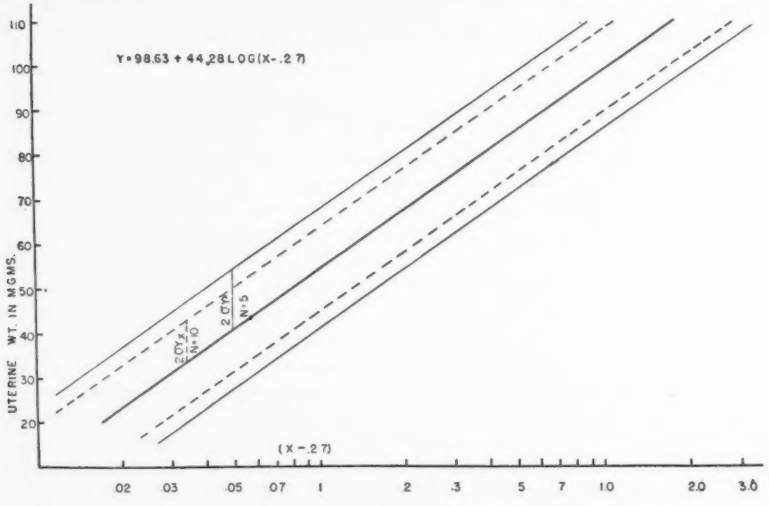

Fig. 2. THE DOSAGE-RESPONSE RELATIONSHIP PLOTTED ON SEMILOG SCALE. The standard errors of estimate for 10 animals (broken lines) and for 5 animals (solid lines) are added.

At the bottom of the curve the rise was very sharp; toward the higher dose levels the uterine weight increase was less for added increments of dose, so that the curve flattened. There was, of course, some spread of the individual weights around the mean, the standard errors of the mean varying from 2.68–7.13 mg.

The standard error of estimate σy_x equals 13.83 mg. if one rat is used for testing. If n rats are used for each dose, the σy_x becomes $\sqrt{n-1}$ times smaller. It is

TABLE 1

Mean weight of uteri	Estimated dosage[1] of gonadotropin	Limits within which the true dosage may be expected 95% of the time			
		No. animals=5		No. animals=10	
		Lower	Upper	Lower	Upper
mg.	I.S.U.				
30	0.46	0.43	0.50	0.44	0.49
40	0.49	0.44	0.56	0.46	0.53
50	0.53	0.47	0.65	0.49	0.61
60	0.61	0.52	0.84	0.53	0.74
70	0.76	0.58	1.11	0.62	0.97
80	0.99	0.68	1.59	0.76	1.37
90	1.38	0.88	2.40	1.00	2.00
100	2.04	1.20	3.77	1.40	3.10

[1] The dosages have been converted to International Standard units for convenience in use.

6.92 mg. with 5 rats, 4.62 mg. with 10 rats. The estimated dosage $\pm 2\sigma y_z$ gives the limits within which one may expect the true value 95% of the time (fig. 2, table 1).

The ovarian weights showed a very slow rise, with a range between control weight of 12.9 mg., and maximum dose weight of only 26.7 mg. Corpora lutea were present in two cases in the 4.0 mg. group, but there were none at any lower level at 72 hours.

The chart for vaginal cornification indicates that its use has limitations. At the lower dosage levels, represented by the region below the broken line in figure 1, smears did not show cornified cells, but a number showed mucoid fluid or beginning mucosal changes by microscopic examination. However, when full cornification oc-

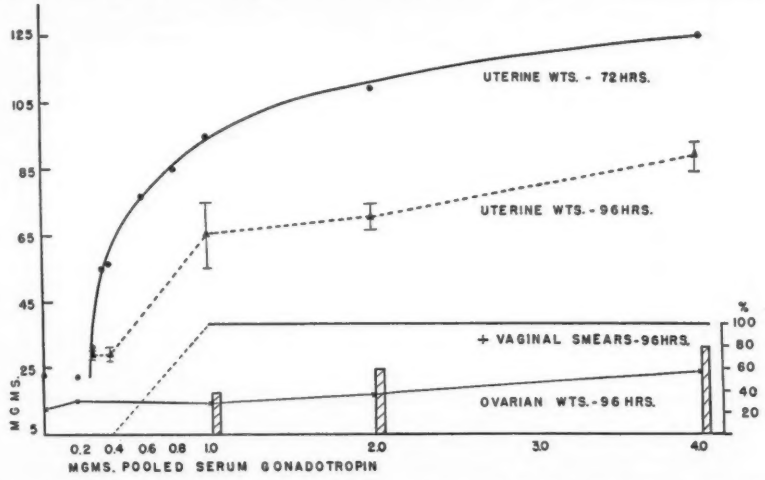

Fig. 3. The 96-hour response of uterine weight, ovarian weight, corpus luteum formation and vaginal cornification to graded doses of pooled serum gonadotropin. The plotted weight is the mean of all of the animals at each dosage level. Vaginal cornification and corpus luteum formation (barred blocks) are represented as percentage of animals showing the response. The 72-hour uterine weight curve is added for comparison.

curred it was at once almost 100%, and hence was of no further use in quantitating an unknown.

Since the 96-hour test period has been so widely used for assays of chorionic gonadotropin, several similar groups of immature rats were given the same dosages and autopsied after 96 hours. In figure 3 these results are compared with the 72-hour uterine weight curve. The most striking difference was in the uterine weight curve, which fell far below that at 72 hours, The minimum effective dose, which gave 150% uterine weight increase at 72 hours, showed a scarcely significant increase at 96 hours. The spread of individual observations was particularly wide at the 1.0 mg. dose level, at which point corpus luteum formation began. Although an increasing percentage of animals at higher levels showed corpora, the increase was so small that one would not be justified in differentiating between points in this region. Ovarian weights showed a slow increase at 96 hours.

During the progress of this work the International Standard chorionic gonadotropin became available. This was assayed at several dose levels by the same technic, and the uterine weight response to the standard material was superimposed on the assay standard curve for pooled serum (fig. 4). The curve for the International Stand-

ard agreed closely and serum assays may be expressed in international units. In case the current International Standard should not prove permanent, results for the curve may be expressed in terms of a defined biological unit, e.g., 100% increase in uterine weight, as used for castrate urine by Levin and Tyndale. By comparison with the International Standard the potency of the pooled serum was 11,500 I.U. per liter. This is an average value for serum gonadotropin in 100 normal women in the last month of pregnancy.

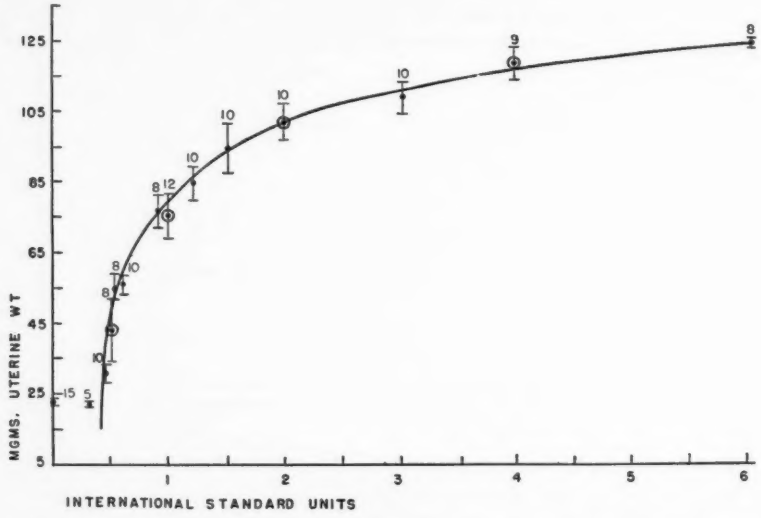

Fig. 4. THE 72-HOUR UTERINE WEIGHT RESPONSE CURVE FOR SERUM GONADOTROPIN STANDARDIZED IN UNITS OF INTERNATIONAL STANDARD CHORIONIC GONADOTROPIN. The circled points are assays of International Standard chorionic gonadotropin, the others serum gonadotropin. Standard error and number of animals are indicated for each point.

In actual use of the assay curve with unknown material, results check well. In 9 unknowns chosen at random, in which repeat assays at the same dosage were made, using 2 to 5 animals, or in which 2 or more different dosages fell on the useful range of the curve, independent values for the unknown varied less than 10% in most cases (table 2). If the approximate potency of the material to be tested is known, assay with 3 to 4 animals at one dose level is usually sufficient. If the unknown potentially may fall within a very wide range, one may reduce the animals used by running a preliminary assay with one animal at each level at 5 to 10-fold intervals; accurate assay is then completed with 3 to 4 animals at one dose level in the appropriate range.

DISCUSSION

From a comparison of the curves, it can be seen that the 72-hour uterine weight curve is more sensitive to small amounts of chorionic gonadotropin and more responsive to increments of dosage than the uterine weight at 96 hours, or than changes in ovaries and vagina at either time interval.

Considering only the steep portions of the curve between the minimum effective dose and a 7-fold increase, the uterine weight increase ranged from 56 mg. to 118 mg. The average uterine weight for the control group was 22.7 mg., so that the observed weights increased approximately 150 to 420%. These differences are sufficiently large

to diminish the importance of technical errors and, to some extent, of individual variation in animal response. It was found that much more uniform results were obtained with 3 injections daily; when the same total dosage was given in fewer injections more variation in response occurred. This is in accordance with the observations of Hamburger and Pedersen-Bjergaard (19) with chorionic gonadotropin and of Heller, Lauson and Sevringhaus (15) with pituitary gonadotropin. The close spacing of injections over the first 2 days gives a longer period following the total dosage for development of the secondary uterine effect.

The ovarian weight curves rose very slowly with either the 72 or 96-hour test period. The percentage of corpora lutea at 96 hours increased progressively but

TABLE 2. CHORIONIC GONADOTROPIN ASSAYS ON 9 SERUM SPECIMENS

Case	No. animals	I. U. per Liter	Weighted mean	Variation from mean	Case	No. animals	I. U. per Liter	Weighted mean	Variation from mean
				%					%
G.R.2	2	270,000	281,000	3.9	N.G.1	2	15,360	17,720	7.1
	2	292,000		3.9		3	19,300		8.9
G.R.3	3	174,000	169,400	2.7	N.G.2	5	11,470		9.9
	2	162,500		4.0		3	12,650	12,730	0.6
						3	14,930		17.2
McK.3	2	10,930	12,472	12.3	Br.	3	13,440		10.4
	3	13,500		8.2		3	15,360	15,005	2.4
McK.4	2	2,550	2,675	4.7		3	14,560		2.9
	2	2,800		4.7		3	16,660		11.0
M.C.	4	10,600	11,300	6.2	I.J.	2	13,800	15,240	9.4
	4	12,000		6.2		3	16,200		6.3

slowly over such a wide dosage range that a large number of animals would be required for accurate evaluation. Corpus luteum formation occurred only at high dosages at 72 hours. In assaying unknown material, it should be noted that the association of marked ovarian stimulation and increased uterine weight indicates that the dosage has exceeded the useful range of the curve.

Vaginal cornification proved to be almost an all-or-none response, hence for assay pusposes could be used only as an end-point in the determination of a minimal effective dose.

Gonadotropic assay of serum has some advantages over urine for routine use (other than studies of excretion), as a single specimen is a more accurate measure of circulating gonadotropin. Single urine specimens are subject to variation, due to fluctuation in the amount and concentration of urine at different times of day. Collection of total specimens is often troublesome and, unless special precautions are taken, there may be some loss of activity during the collection period.

The question may be raised as to whether the uterine weight assay method differentiates satisfactorily between gonadotropin of chorionic origin and that of pituitary origin. Van Dyke, Greep and Chow (20) have showed that pure FSH does not produce uterine enlargement. As is well known, cruder material, such as extracts of urine of castrates and post-menopausal women, does produce uterine weight increase and this has been used as an assay endpoint for these gonadotropic substances (14). With such material, the uterine changes are generally associated with marked ovarian weight increase, with a minimum response ratio of 1:2 or even 1:1, according to Heller and Heller (21); however, with chorionic gonadotropin, the useful range of

the uterine weight curve falls below the level of significant ovarian weight increase. For this reason the ovaries are weighed with each assay. From a practical standpoint, this problem is not often important, as the serum concentration of pituitary gonadotropin rarely exceeds one unit in 40 cc., and probably never exceeds one unit in 10 cc. The chorionic gonadotropin assays are carried out with a small fraction of 1 cc. of serum, only occasionally as much as 1 to 2 cc. being used. If the uterine weight method is used for assay of urines when the titre may be high or the nature of the gonadotropin may be in question, the cresol extraction method of Fevold and Hisaw (22) may be used, as this extracts only the chorionic gonadotropin.

SUMMARY

An assay method is presented for human serum chorionic gonadotropin, based upon the 72-hour uterine weight response of the immature rat. Female animals of 21 to 23 days have been given 6 injections spaced over 2 days and the uterine weight increase determined at 72 hours. A standard curve for dosage—uterine weight response has been developed, which is accurate over a 7-fold dosage. Comparison with International Standard chorionic gonadotropin has been made and results can be expressed in international units. This method, with relatively few animals, has been found to be more sensitive and to have a wider useful range than other commonly used end-points.

The author is indebted to Drs. E. L. Crosby and M. Cakrtova of the Department of Statistics for assistance in statistical analysis of the data.

REFERENCES

1. Delfs, E.: *Proc. Am. Physiol. Soc.* March, 1940.
2. Smith, P. E.: *Am. J. Physiol.* 80: 114. 1927.
3. Severinghaus, A. E.: *Am. J. Physiol.* 101: 309. 1932.
4. Siddall, A. C.: J.A.M.A. 90: 380. 1928.
5. Siddall, A. C.: J.A.M.A. 91: 779. 1928.
6. Emory, F. E., P. W. Bash and W. R. Lewis: *Proc. Soc. Exper. Biol. & Med.* 29: 42. 1931.
7. Fluhmann, C. F.: *Proc. Soc. Exper. Biol. & Med.* 29: 1193. 1932.
8. Kennedy, W. P.: *Quart. J. Exper. Physiol.* 23: 373. 1933.
9. Davy, L.: *Proc. Soc. Exper. Biol. & Med.* 32: 927. 1935.
10. Cahen, R., and P. Ardoint: *Compt. Rend. Soc. de Biol.* 123: 547. 1936.
11. Cahen, R., and P. Ardoint: *Compt Rend. Soc. de Biol.* 130: 949. 1939.
12. Owen, S. E., and L. H. Prince: *J. Lab. & Clin. Med.* 22: 431. 1937.
13. D'Amour, M. C., and F. E. D'Amour: *J. Pharmacol. & Exper. Therap.* 62: 263. 1938.
14. Levin, L., and H. H. Tyndale: *Endocrinology* 21: 619. 1937.
15. Heller, C. G., H. Lauson and E. L. Sevringhaus: *Am. J. Physiol.* 121: 364. 1938.
16. Hamburger, C., and K. Pedersen-Bjergaard: *Quart. J. Pharmacol. and Pharmacol.* 10: 662. 1937.
17. D'amour, F. E., and M. C. D'Amour: *Endocrinology* 26: 93. 1940.
18. Sealy, J. L., and C. W. Sondern: *Endocrinology* 26: 813. 1940.
19. Hamburger, C., and K. Pedersen-Bjergaard: *Quart. J. Pharmacol. & Pharmacol.* 11: 186. 1938.
20. Van Dyke, H. B., R. O. Greep and B. F. Chow: *Proc. Am. Physiol. Soc.* Annual Meeting, March, 1940.
21. Heller, C. G., and E. J. Heller: *Endocrinology* 24: 319. 1939.
22. Fevold, H. L., and F. L. Hisaw: *Proc. Soc. Exper. Biol. & Med.* 34: 712. 1936.

COMPARATIVE BIOLOGIC RESPONSES TO THE INTERNATIONAL STANDARD AND COMMERCIAL CHORIONIC GONADOTROPINS

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THE AVAILABILITY of an established standard for chorionic gonadotropin (the designation for the gonadotropic principle from human pregnancy urine—Bulletin of the Health Organization of the League of Nations (1)) should materially assist in establishing and maintaining uniformity both as to potency and stability of commercially available preparations.

The recommendations for the use of the above standard for the comparative determinations of gonadotropic activity in units on immature female rats depends upon the observation of a direct or indirect gonadotropic effect, shown by morphological changes in the gonads, and the observations of secondary changes in the accessory reproductive organs, in animals not deprived of their gonads. When this type of test is used, the absence of substances causing such changes in the accessory reproductive organs should be assured by control tests on animals deprived of their gonads.

On the basis of these recommendations, which have been adopted by the U. S. P. Advisory Committee on Hormones, it is possible to assay a gonadotropic preparation from pregnancy urine on immature rats of any age and the endpoints of the assay may be based on one or more of several criteria, as long as the responses to the standard are reproduced by the material under assay. The reliability of various assay methods have been indicated in excellent comparative studies of the International Standard and commercial preparations by D'Amour and D'Amour (2, 3). The need for a standard test method, in addition to the International Standard for the hormone, is illustrated by the published Summary of Biological Standardization studies by collaborative laboratories through the League of Nations Bulletin of the Health Organization (4) in which the following statements appear:

> Different test methods also show significantly different degrees of accuracy. The methods employing vaginal cornification and ovarian weight respectively were the most accurate of those for which adequate data were available. Uterine weight was the least accurate criterion.

However, the dosage of the standard required to give a 50% increase in the weight of the ovaries with corpora lutea varied in different test laboratories from about 3 to 50 mg.

Since, "the specific gonadotropic activity of 0.1 mg. (100 gamma) of the standard preparation shall be the international unit for recording the activities of all gonadotropic preparations of human urine of pregnancy" (excerpt from above Bulletin), the advisability of establishing uniform assay procedures is apparent.

Received for publication July 29, 1940.

The present study permits a comparison of the International Standard for chorionic gonadotropin and the initial potency and stability with time under different storage conditions of various commercial preparations of the hormone by uniform assay methods on immature rats of different ages by the several criteria available.

METHOD OF ASSAY

The assays were carried out on normal immature female rats from a single colony (Winthrop) unless otherwise indicated. In a single series of tests of the International Standard 21-day-old rats were used (table 1). In all remaining assays 26- to 28-day-old rats were used. Such animals weighed 45 to 55 gm. at the beginning of the assay period.

Dilutions of the products under test were made in normal saline (unless directed otherwise on the package insert), so that the total dose for each rat was contained in 1.5 cc. The total dose was injected subcutaneously in 6 divided doses of 1/4 cc.

TABLE 1. CHORIONIC GONADOTROPIN ASSAYS ON 21-DAY-OLD RATS

Dose	No. of rats	Body weights Initial	Body weights Final	Estrous smears	Uterine weight Range	Uterine weight Average	Ovarian weight Range	Ovarian weight Average	Corpora lutea
		gm.	gm.	%	mg.	mg.	mg.	mg.	%
Controls	30	34.3	43.5	None	15–32	21.35	8–17	11.4	None
International Standard									
½ I.U.	20	36.4	51.3	None	16–26	21.3	8–19	12.5	None
1 I.U.	20	36.9	50.7	50	20–64	34.8	5–16	10.5	None
2 I.U.	25	36.9	50.9	88	46–91	65.0	7–17	11.7	20
4 I.U.	25	34.6	47.9	100	61–93	71.1	8–25	15.1	62
Korotrin Lot EO 391									
½ I.U.	10	35.0	44.4	None	15–32	25.8	5–14	10.0	None
1 I.U.	15	35.4	49.2	53.5	21–43	33.7	5–19	12.1	None
2 I.U.	15	36.8	50.9	80.0	42–84	63.1	10–21	15.1	26.7
4 I.U.	15	37.0	51.7	100	56–88	67.0	14–27	17.0	60

each at spaced intervals over a period of 44 hours, i.e., the 5th and 6th injections were given on the 3rd day of test. Animals (in groups of 10 per dose) were sacrificed 116 to 118 hours after the first injection.

An examination of the vagina was made and vaginal smears were obtained immediately before killing the animals. Such smears were stained with hematoxylin and eosin and examined microscopically for determination of the stage of the estrous cycle.

At necropsy, the uterine horns and ovaries were dissected out, carefully freed of extraneous tissue and fluid by gentle pressure and sponging and weighed. The presence or absence of blood points (hemorrhagic follicles) was determined by gross examination with a binocular microscope. The incidence of corpora lutea was determined by histological examination of stained sections of the ovaries.

Preparations Examined

The most recently manufactured lots obtainable were purchased directly from large prescription pharmacies in New York City. In one instance (korotrin) additional special trade packages of material from the same lots as purchased were obtained directly from the manufacturer in order to note the stability of such a product over a prolonged period of time.

The products tested included the following.

1. Antuitrin-S, Lot #3226754 (Parke, Davis & Co.) manufactured 2/8/39—10 cc. vial —labeled to contain 100 rat units per cc.

2. Antuitrin-S, Lot #3240332 (Parke, Davis & Co.) manufactured 6/19/39—10 cc. vial—labeled to contain 100 rat units per cc.
3. Antuitrin-S, Lot #3270287 (Parke, Davis & Co.) manufactured 2/22/40—10 cc. vial—labeled to contain 100 rat units per cc.
4. A.P.L. Lot *R 10070* (Ayerst, McKenna & Harrison)—10 cc. vial—labeled to contain 500 rat units (Collip) per cc.
5. A.P.L. Lot *R 10268* (Ayerst, McKenna & Harrison)—10 cc. vial—labeled to contain 100 International units per cc.
6. A.P.L. Lot *R 11160* (Ayerst, McKenna & Harrison)—10 cc. vial— labeled to contain 100 International units per cc.
7. Follutein Lot #66093 (E. R. Squibb & Co.)—10 cc. vial—containing the hormone concentrated in glycerine to which sterile distilled water is added to volume at time of use. Labeled to contain (when so diluted) 100 rat units per cc.
8. Follutein Lot #67324 (E. R. Squibb & Co.)—as above.
9. International Standard Chorionic Gonadotropin—National Institute for Medical Research, Hampstead, London, England (Tablets approx. 100 mg.) Each 0.1 mg. = 1 International Unit.
10. Korotrin Lot *EO 391* (Winthrop Chemical Company, Inc.)—vials of dry powder—labeled to contain 5000 International Units.
11. Korotrin Lot *EO 394*—as above.
12. Pregnyl Lot #306028 (Roche-Organon, Inc.)—ampules of dry powder—labeled to contain 500 rat units each.
13. Pranturon Lot 9PB01 (Schering Corporation)—ampules of dry powder— labeled to contain 750 International units.

RESULTS

The influence of the International Standard Chorionic Gonadotropin in a dosage range from ½ to 4 units on the vaginal smear, uterine weights, ovarian weights and

TABLE 2. CHORIONIC GONADOTROPIN ASSAYS—26 TO 28-DAY-OLD RATS[1]

Dose, subcut.	No. of rats	Body weights Initial	Body weights Final	Estrous smears	Uterine weight, average	Ovarian weight, average	Copora lutea, incidence
		gm.	gm.	%	mg.	mg.	%
Controls							
—	40	50	65	0	23.7	14.0	0
International Standard							
1 I.U.	10			80	63.2	15.8	55.0
1 I.U.	10			100	77.2	15.4	45.0
1 I.U.	10			80	53.8	14.8	20.0
1 I.U.	10			100	75.7	21.2	70.0
1 I.U.	10			90	63.1	15.1	65.0
1 I.U.	60			86.7	65.2	16.2	47.5
1 I.U.	200			90	64.4	16.6	40.0

[1] All rats killed at 31–33 days of age.

the incidence of corpora lutea of 21-day-old immature female rats (beginning of test) is illustrated in table 1. These data indicate that the test value of the several criteria on such animals from high to low is of the following order: estrous smear response > uterine hypertrophy > incidence of corpora lutea and least reliable, ovarian hypertrophy. One International Unit of the standard causes an approximate 50% increase in uterine weights and induces estrus in 50% of a series of 20 animals. The production of a definite ovarian hypertrophy requires doses in excess of 2 U. The development of blood points or corpora lutea required a dosage of 2 U and a 60% incidence of blood points followed the administration of 4 I.U. of the standard.

The influence of 1 U of the International Standard on the gonads and accessory reproductive organs of consecutive groups of ten 26- to 28-day-old rats (beginning of test) and of massed assay groups of 10 animals each is illustrated in table 2.

The values of the 4 test criteria from the standpoint of regularity in response have the following order from greatest to least: (*a*) estrous smears, (*b*) uterine hypertrophy, (*c*) incidence of corpora lutea, and (d) ovarian hypertrophy. Although a variation in the degree of response is noticeable in successive tests with a unit dose by all end-points, the order of variability diminishes in the order named. Our data indicate that all routine assays should include minimum numbers of 10 animals per group. Such

TABLE 3

Laboratory or animal source	Age of rats[1]	No. per group	Uterine weight Range	Uterine weight Average	Ovarian weight Range	Ovarian weight Average	Source of data
	days		*mg.*	*mg.*	*mg.*	*mg.*	
Univ. of Denver	25	10–250		15.0–15.4	7.5–16.0	10.2–11.9	F. E. D'Amour
Univ. of Denver	32	200 or more	12.2–22.4	19.3	9.0–15.8	13.6	F. E. D'Amour

"In all rats included above, we have found an open vagina, with, however, no indication of sexual maturity in ovary or uterus and rather think it was mechanically ruptured"(8).

Woodruff & Sons, Detroit	31[2]	20	15–32.5	21.8	8.0–16.5	11.95	
Rockland Farms	32[3]	25	21–31	24.1	7–19	11.7	
Winthrop Chemical Company	26[4]	30	15–32	21.35	8–17	11.4	
	32[5]	40	17–29	23.7	9–16	14.0	

[1] Age at time of killing.
[2] Average weight of 60 animals—26 days (28.7 gm.) 31 days (49.0 gm.).
[3] Initial weights—27 days (43.6 gm.) 32 days (59.75 gm.).
[4] Initial weights—21 days (34.3 gm.) 26 days (43.5 gm.).
[5] Initial weights—27 days (50.0 gm.) 32 days (65.0 gm.).

assays should include animals of uniform weight as well as age, a positive control group and responses to a minimum of 2, and preferably 3, dose levels of preparations of unknown potency should be ascertained.

Our data indicate that 1 U of the International and present U.S.P. standard for chorionic gonadotropin will induce (in 26- to 28-day-old rats at beginning of assay) an estrous smear in at least 8 of 10 animals, a 250 to 300% increase in uterine weights, a 40 to 50% incidence of blood points and/or corpora lutea and a slight ovarian hypertrophy of the order of 10 to 15%.

On the basis of observed responses of significant numbers of animals the 26- to 28-day-old immature rat appears to be not only more sensitive and uniform in responses to the hormone from human pregnancy urine by all 4 criteria used, but also superior in other respects to the 19- to 21-day-old animals. The older rats have a more uniform uterine and ovarian weight as well as body weight to age ratio. The relative sensitivity of immature rats of different ages may be noted on comparing corresponding dose responses as indicated in tables 1 and 2.

The report of D'Amour and D'Amour confirms these differences in certain respects, as to incidence of corpora lutea. However, we are quite unable to confirm their observations made on 21-day-old rats from one rat colony that a one-half unit dose of the standard induces a 500% increase in uterine weights and an 80% inci-

TABLE 4

Source of rats	Age of rats at end of expt.	No. per group	Preparation	Dose	Estrous smears	Uterine weight	Corpora lutea	Ovarian weight
	days			R.U.	%	mg.	%	mg.
Woodruff & Sons Detroit[1]	31	20	Controls	—	0	21.8	0	11.95
	32	10	Antuitrin-S	¾	70	43.1	20	9.2
	32	10	Lot #3226754	1	90	42.6	20	10.6
	32	10	Aged 3 months	2	100	50.6	25	10.5
	32	10	Aged 3 months	3	100	59.0	10	11.0
Winthrop Chemical Company	32	40	Controls	—	0	23.7	0	14.0
	32	10	Antuitrin-S	¾	80	54.6	15	12.6
	32	10	Lot #3226754	1	90	62.8	30	14.3
	32	10	Aged 2 months	1¼	100	68.5	55	17.7
	32	10	Aged 2 months	1½	100	83.0	75	18.8

[1] This colony of rats was used for assay purposes by Bradbury and Gaensbauer—*Endocrinology* 24: 867. 1939.

dence of estrous smears or that a single unit gives a 100% estrous and more than a 600% increase in uterine weights.

The importance of using selected normally growing animals from a single colony is illustrated by the tabulated data for rats from 4 sources as illustrated in table 3. Such differences, however, cannot eliminate at times a rather striking variability of different strains to uniform doses of the standard as shown by comparative responses of our colony (tables 1, 2) with those of the University of Denver, those of Woodruff & Sons (table 4) used by Bradbury and Gaensbauer (5), or those from the Rockland Farms (table 5), and emphasize the basic requirement of a positive control group on each assay.

Potency of Commercial Preparations

A comparison of the potency of one or more lots of several commercial chorionic gonadotropin preparations purchased on the open market with the International Standard on initial tests and at intervals thereafter is illustrated in table 6.

In the case of the preparations in solution form, precautions were taken to maintain sterility. All stock preparations were maintained at 4°C. during the period of examination.

TABLE 5. RESPONSES OF 26 TO 28-DAY-OLD RATS (ROCKLAND FARMS) TO CHORIONIC GONADOTROPIN

Preparation	Dose	No. of rats	Body weight Initial 27 days	Body weight Final 32 days	Estrous smears	Uterine weight	Ovarian weight	Corpora lutea
	I.U.		gm.	gm.	%	mg.	mg.	%
Controls	—	25	43.6	59.7	—	24.1	11.7	—
International Standard	¼	40	43.6	62.3	33.3	46.5	12.7	6.7
	½	40	44.8	61.8	90.0	60.0	14.3	43.8
	¾	40	42.1	57.1	100.0	59.8	15.9	87.5
	1	40	46.6	61.8	100.0	58.0	18.0	90.3
Korotrin *EO* 391	¼	10	42.3	57.5	40.0	51.6	14.3	—
	½	10	44.0	59.7	90.0	58.5	15.8	60.0
	¾	10	43.9	60.4	100.0	70.4	18.4	85.0
	1	20	45.4	62.1	100.0	64.2	18.2	95.0

These data indicate that 3 lots of antuitrin-S tested within 1½ to 2½ months after manufacture were approximately equivalent in potency to the International Standard. One lot, #3240332, deteriorated 50% within 5 months and approximately 66% within 6½ months after manufacture on the basis of estrous smears, uterine hypertrophy and incidence of corpora lutea responses.

Tests on A.P.L. Lot R10070 indicated that 6 Collip rat units correspond to slightly less than one International unit of hormone. The observed potency claims for

TABLE 6. COMPARATIVE ASSAYS OF COMMERCIAL PREPARATIONS AND CHORIONIC GONADOTROPIN

Date tested	Product	Lot No.	Date of mfg.	Age	Dose on basis of label	No. of rats	Estrous smears	Uterine weight	Corpora lutea	Ovarian weight
				mo.			%	mg.	%	mg.
Aug./Sept. 1939	Int. Standard	—	—	—	1 I.U.	30	83.3	66.1	46.7	15.4
Nov./Dec. 1939	Int. Standard	—	—	—	1 I.U.	30	90.0	64.2	50.0	17.0
April 1939	Antuitrin-S	#3226754	2/8/39	2	1 R.U.	10	100	85.0	70	18.8
Aug. 1939		#3240332	6/19/39	2½	1 R.U.	20	85	64.3	35	15.8
Nov. 1939		#3240332	6/19/39	5	1 R.U.	10	30	43.0	0	12.0
Nov. 1939		#3240332	6/19/39	5	1 R.U.	10	40	49.3	0	13.8
Nov. 1939		#3240332	6/19/39	5	2 R.U.	10	80	58.4	45	15.6
Nov. 1939		#3240332	6/19/39	5	4 R.U.	10	100	69.2	90	19.6
Jan. 1940		#3240332	6/19/39	6½	2 R.U.	10	80	56.8	10	11.1
Jan. 1940		#3240332	6/19/39	6½	3 R.U.	10	100	78.8	50	15.2
April 10, 1940		#3270287	2/22/40	1½	½ R.U.	10	80	54.6	15	12.6
April 10, 1940		#3270287	2/22/40	1½	1 R.U.	10	90	62.8	30	14.3
April 10, 1940		#3270287	2/22/40	1½	1½ R.U.	10	100	68.5	55	17.7
April 10, 1940		#3270287	2/22/40	1½	1½ R.U.	10	100	83.0	75	18.8
Aug./Sept. 1939	A.P.L.	R10070	—	—	6 R.U.	10	10	44.2	0	14.1
Aug./Sept. 1939		R10070	—	—	6 R.U.	10	70	61.9	30.0	16.0
Nov. 1939		R10070	—	—	8 R.U.	10	100	63.1	65	15.1
Jan. 4, 1940		R10070	—	4½	8 R.U.	10	90	71.3	40	12.0
Aug. 1939		R1026A	—		1 I.U.	10	90	68.2	45	15.7
Nov. 1939		R1026A	—		1½ I.U.	10	100	80.6	45	17.6
Jan. 4, 1940		R1026A	—	4½	1½ I.U.	10	100	73.6	60	15.3
Jan. 4, 1940		R1026A	—	4½	2 I.U.	10	90	77.7	80	15.5
April 8, 1940		R11160	Expir. 7/1/40		1 I.U.	20	30	40.4	0	14.8
April 8, 1940		R11160			1½ I.U.	20	70	46.8	15	14.8
April 8, 1940		R11160			2 I.U.	10	100	70.2	50	14.7
April 8, 1940		R11160			3 I.U.	10	100	78.4	70	18.7
Aug. 1939	Follutein	#66093	—	—	1 R.U.	10	90	67.5	35	15.6
Nov. 1939		#66093	—	3	1½ R.U.	10	60	61.9	20	15.4
Aug. 1939		#67324	—	—	1 R.U.	10	60	53.7	0	12.0
Aug. 1939		#67324	—	—	1½ R.U.	10	90	76.2	60	16.3
Nov. 1939		#67324	—	3	1½ R.U.	10	80	69.9	30	17.7
July 1938	Korotrin	*EO* 391	—	2½	1 R.U.	20	100	65.6	45	16.1
Aug. 1939		*EO* 391	—	15	1 I.U.	20	100	68.4	60	17.6
Jan. 1940		*EO* 391	—	20	1 I.U.	20	100	66.3	55	16.6
Jan. 1940		*EO* 391	—	25	1 I.U.	20	100	62.4	45	15.9
Aug. 1939		*EO* 394	—	9	1 I.U.	20	100	62.0	40	16.1
Nov. 1939		*EO* 394	—	12	1 I.U.	20	100	66.4	50	17.5
Nov. 1939	Pranturon	9PB01	—	—	1 I.U.	20	100	62.4	55	17.3
Aug. 1939	Pregnyl	#306028	—	—	1 R.U.	10	90	59.8	30	13.0
Aug. 1939		#306028	—	—	1½ R.U.	10	100	68.5	85	18.9

Lot R1026A were satisfactory and no deterioration was apparent 4½ months after manufacture. The potency of Lot R11160 on the contrary appeared to be between 25 and 50% below labeled claims.

The two lots of follutein tested at a 1 R.U. level were distinctly different in potency. Responses to 1 R.U. of Lot #66093 roughly corresponded to that of 1 I.U. On the other hand Lot #67825 was somewhat below claims. Storage of the water glycerine solution of the hormone under sterile conditions at 4°C. for 3 months resulted in a slight deterioration.

Repeated assays of chorionic gonadotropin in powder forms, i.e., International Standard and korotrin Lots *EO* 391 and *EO* 394, over periods of 25 to 12 months have indicated no changes in potency over the period of observation. Pranturon was

likewise found to be satisfactory. The pregnyl R.U. on the basis of the estrous smear equalled 1 I.U., but corresponding uterine changes, ovarian hypertrophy and incidence of corpora lutea were slightly inferior to those of unit doses of the standard.

Influence of incubation on the stability of chorionic gonadotropin. The stability of 3 commercial preparations—1 powder (korotrin) and two solutions—of chorionic gonadotropin under incubator conditions (37.5°C.) with time is illustrated in table 7. With each preparation separate packages of a single lot were used for each test in order to avoid possible contamination. A single bottle of each preparation was removed from the incubator at each period, tested and kept in a refrigerator at 4°C. during and following each assay.

The potency of chorionic gonadotropin in powder form was not impaired after

TABLE 7. INFLUENCE OF INCUBATION (37.5°) ON STABILITY OF CHORIONIC GONADOTROPIN

Preparation	Lot No.	Package date	Date assayed	Period of inc. days	Dose	No. of rats	Estrous smears	Uterine weight	Corpora lutea	Ovarian weight
							%	mg.	%	mg.
Korotrin	EO 391	None	6/5/40	None	1 I.U.	20	100	62.4	45	15.9
		None	5/9/40	22	1 I.U.	10	100	60.4	40	16.1
		None	5/9/40	22	1½ I.U.	10	100	79.4	80	20.0
		None	6/5/40	49	1 I.U.	10	100	55.8	25	15.8
		None	6/5/40	49	1½ I.U.	20	100	74.4	55	16.0
A.P.L.	R11160	Exp.	4/8/40	None	1 I.U.	20	30	40.4	0	14.8
		7/1/40		None	1½ I.U.	10	70	46.8	15	14.8
		7/1/40		None	2 I.U.	20	100	70.2	50	14.7
		7/1/40		None	3 I.U.	10	100	78.4	70	18.7
		7/1/40	4/24/40	9	2 I.U.	10	100	62.4	30	15.9
		7/1/40	4/24/40	9	3 I.U.	10	100	79.6	60	18.3
		7/1/40	5/2/40	17	2 I.U.	10	20	39.8	0	14.0
		7/1/40	5/2/40	17	4 I.U.	10	100	77.8	60	18.0
		7/1/40	5/2/40	17	8 I.U.	10	100	81.4	80	23.4
		7/1/40	5/9/40	24	2 I.U.	10	0	30.4	0	13.9
		7/1/40	5/9/40	24	4 I.U.	10	100	68.4	40	15.6
		7/1/40	5/9/40	24	6 I.U.	10	100	74.8	60	19.6
		7/1/40	6/5/40	51	4 I.U.	10	60	53.4	0	14.6
		7/1/40	6/5/40	51	8 I.U.	10	100	83.4	40	19.0
		7/1/40	6/5/40	51	12 I.U.	10	100	79.8	90	19.5
Antuitrin-S	#3270287	Mfg.	4/10/40	None	½ R.U.	10	80	54.6	15	12.6
		2/22/40		None	1 R.U.	10	90	62.8	30	14.3
		2/22/40		None	1½ R.U.	10	100	68.5	55	17.7
		2/22/40		None	1½ R.U.	10	100	83.0	75	18.8
		2/22/40	4/24/40	9	1½ R.U.	10	0	23.8	0	13.8
		2/22/40	4/24/40	9	2 R.U.	10	40	31.6	0	14.4
		2/22/40	5/2/40	17	4 R.U.	10	0	25.4	0	15.6
		2/22/40	5/2/40	17	12½ R.U.	10	100	54.0	40	17.6

22 days incubation. After 49 days incubation a questionable deterioration of the order of 15 to 20% may have occurred, although the assay results obtained were within the lower limit of successive tests of the standard.

The hormone value of A.P.L. Lot R11160 on the basis of control tests was not definitely impaired after 9 days incubation, but diminished materially after 17, 24 and 51 days incubation. The deterioration at these intervals was of the order of 40 to 45, 50 to 60 and 75 to 80%.

Antuitrin-S Lot #3270287 tested 1½ months after manufacture deteriorated under incubator conditions more than 50% in 9 days and approximately 90% in 17 days.

SUMMARY

On the basis of assays of the International Standard for Chorionic Gonadotropin from human pregnancy urine, it appears that the 26- to 28-day-old immature normal female rat at the time of initial injection responds more strongly and uniformly than 21-day-old rats to unit dosages.

A comparison of the responses to the International Standard by 4 different assay criteria indicates that the incidence of estrous smears, degree of uterine hypertrophy, incidence of blood points or corpora lutea, and ovarian hypertrophy are of decreasing test value in the order named. A combined criteria of 80% or more estrous smears with a 250 to 300% increase in uterine weights appears to be the most reliable index of a unit response to the standard.

Parallel observations as to the incidence of blood points and corpora lutea as well as the degree of ovarian hypertrophy aid materially in comparative assays of different products. The latter two criteria taken alone as an assay basis are unreliable.

Satisfactory biological assays of preparations of chorionic gonadotropin require not less than 10 test animals per dose and results should be based on responses to a minimum of 2, or preferably 3, dosage levels. Assay results should be uniformly controlled by responses to a standard unit. The use of normally growing animals of equal age and weight from a particular rat colony contribute materially to the uniformity of assays. Animals from different colonies may vary widely as to growth rates, reproductive organ weights and sensitivity to the hormone.

The procedures used give a satisfactory comparative assay of various products and on the basis of stability tests at 4°C. and 37.5°C. indicate that the hormone in powdered form is superior to that of solutions.

REFERENCES

1. *Bull. Health Organization of the League of Nations* 7: 889. 1938.
2. D'AMOUR, F. E.: *Endocrinology* 22: 683. 1938.
3. D'AMOUR, F. E.: *Endocrinology* 26: 88. 1940.
4. *Bull. Health Organization of the League of Nations* 8: 897. 1939.
5. GAENSBAUER, F., AND J. T. BRADBURY: *Endocrinology* 24: 867. 1939.
6. EMMENS, C. A.: *Bull. Health Organization of the League of Nations* 8: 862. 1938.
7. D'AMOUR, F. E., AND M. C. D'AMOUR: *Endocrinology* 26: 93. 1940.
8. D'AMOUR, F. E.: Personal communication.

ARE RESULTS OF GONADOTROPIC ASSAY PERFORMED ON THE INTACT IMMATURE RAT VALID?

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THE OBJECT of this investigation was to determine whether or not assays obtained on the intact, immature rat give a valid quantitative evaluation of gonadotropic factors. Some investigators have claimed that the growing follicle of the test animal which produces estrogen, in turn acts on the animal's hypophysis, thereby stimulating the hypophysis to secrete a luteinizing factor (1). This luteinizing factor elaborated by the test animal would then in turn react upon the animal's ovary, causing a luteinization which might be ascribed falsely to the direct action of the injected gonadotropic extract.

There are no data available as to the amount of estrogen secreted by the growing follicles of the test animal when stimulated by gonadotropic injections. There are no data as to the length of the latent period which must elapse between the stimulation of the immature ovary and the first secretion of estrogen by it. It appeared to us that the following method of experimentation most closely reproduced the conditions which might be expected to arise in the test animal (rats weighing 30 gm. at the beginning of the experiment).

Preliminarily by injecting groups of animals with ascending doses of the same urinary gonadotropic extract obtained from a surgically castrated woman, the amount necessary to produce luteinization was determined.[1] As seen from table 1, the threshold lies within the dosage range equivalent to 35 to 40 cc. of urine. It was found that the percentage of luteinization increased progressively until it reached 100% with quantities equivalent to 150 cc. of urine. Sole recession in this trend was noted at 50 cc.

On the basis of these preliminary data, two groups of animals were injected, each with equivalents of 40 cc. of the same gonadotropic factors. The first group was primed with ascending doses of estrogen (progynon-B), 48 hours before beginning injections with gonadotropic material. After the elapse of 48 hours these animals were then injected in the usual way, i.e., 5 injections in the course of 60 hours, with the equivalent of 40 cc. of the gonadotropic powder and autopsied hours 96 after the first gonadotropic injection.

The second series of animals received identical ascending doses of estrogen, and the equivalents of 40 cc. of gonadotropic urine factor, but the injections were given simultaneously. The animals were autopsied 96 hours following the first injection. The first group (those receiving estrogens 48 hours in advance) were designated as 'primed'; the second group as 'simultaneous.'

In the preliminary group in which 40 cc. of gonadotropic factor alone was injected (table 1), the ovaries of 12% of these animals were found luteinized. In the

Received for publication August 15, 1940.

[1] Titration on castrated mice showed no estrogenic activity in the powder (the method used for obtaining the powder was the modified Zondek alcohol method, *Endocrinology* 25: 996. 1939).

primed group, an increase in luteinization occurred. This luteinization increased progressively with quantities of 0.5, 1.0, and 1.5 R.U. of estrogen. Injections of quantities above this were accompanied by a definite regression in percentage of luteinization. In the 'simultaneous' group no increase in luteinization was observed with 0.5 R.U. of estrogen, but some increase occurred with 1.0 and 1.5 R.U. However, above these quantities, a greater increase was noted, reaching a plateau corresponding to that noted in the 'primed' group.

TABLE 1.

Amount of urine		Number of luteinized ovaries	Number of animals in group	% luteinization	
cc.		*G. H. Equivalent*			
20		0	15	0	
35		0	10	0	
40		3	25	12	
45		3	10	33	
50		2	10	20	
60		6	15	40	
80		11	15	73	
100		12	15	80	
150		15	15	100	
'Primed' group receiving estrogen 48 hours before gonadotropic factor was injected					
Estrogen	G.H.				
R.U.	cc.				
0.5	40	4	11	36	58%
1.0	40	3	5	60	
1.5	40	8	10	80	
2.0	40	5	10	50	
5.0	40	2	7	26	
25.0	40	3	7	43	
'Simultaneous' group, estrogen and gonadotropic factor injected simultaneously					
0.5	40	1	10	10	16.6%
1.0	40	2	10	20	
1.5	40	2	10	20	
2.0	40	3	10	30	
5.0	40	4	10	40	
25.0	40	4	10	40	

From a priori reasoning, it seems justified to believe that the autosecretion of estrogen of the gonadotropically stimulated ovaries of the test animal should lie below the limit of 1.5 R.U. and therefore our attention has been focused on a group of 'simultaneous' animals in which 0.5, 1.0 and 1.5 R.U. were injected. The mean percentage of luteinization of these 30 animals was found to be 16.6%, in contrast to 58% in the similar 'primed' group of 25 animals in which the estrogen had had 48 hours to act before the gonadotropic injections were started. In the group of 'primed' animals receiving 2.0, 5.0 and 25.0 R.U. respectively, a recession of the percentage of luteinization of the ovaries was noted. This recession may be ascribed to the well established observation that large doses of estrogen produce regressive hypophyseal changes which manifest themselves morphologically by cytological changes in the cells (2) and functionally by reduction in gonadotropic secretion (3). In the 'simultaneous' group, such recession was not noted. If estrogen alone is injected, no ovarian response is obtained in any of the dosage ranges used by us (0.5 R.U. to 25 R.U.).

From these experiments we conclude that the results obtained with intact, immature rats by means of gonadotropic extract containing both follicle stimulating and

luteinizing factors, may be accepted as valid. The difference in percentage of luteinization obtained in the 40 cc. gonadotropic control group (12%) and the 'simultaneous' group (16.6%) appears too small to influence significantly the results in view of the unavoidable percentage error inherent in any method of bioassay. The results obtained in the 'primed' group appear to confirm the observation of those investigators who ascribe to estrogens a stimulating effect upon secretion of the luteinizing factor of the hypophysis, but this effect does not appear within the 96 hours of the bioassay, nor within the dosage range of estrogen presumably produced by the animal's own growing follicle, and is not elicited by estrogenic injections unless gonadotropic factor is added.

REFERENCES

1. Fevold, H. L., F. L. Hisaw and R. Greep.: *Am. J. Physiol.* 114: 508. 1935–1936.
2. Wolfe, J. M., and A. W. Wright: *Endocrinology* 23: 200. 1938.
3. Salmon, U. J., and R. T. Frank: *Proc. Soc. Exper. Biol. & Med.* 33: 612. 1936.
4. Frank, R. T., M. A. Goldberger, and U. J. Salmon: *N. Y. State J. Med.* 36: 1363. 1936.

GONADOTROPIC AND AUGMENTATIVE EFFECTS OF TESTOSTERONE PROPIONATE IN IMMATURE FEMALE RATS[1]

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A NUMBER of reports from various investigators (1–6) have indicated that testosterone has a gonadotropic effect on the ovaries of immature rats. The object of this communication is to record (*a*) the effect of increasing doses of testosterone propionate on the weights of the ovaries and uteri of immature female rats, and (*b*) the results of the preliminary administration of testosterone to animals receiving various gonadotropins.

METHODS

The rats employed in this study were immature female albinos 21 to 23 days of age at the beginning of each experiment. All were offsprings of the inbred colony which has been maintained at Stanford for many years.

The testosterone propionate employed was the preparation manufactured by Ciba under the name of perandren, made up in sesame oil and containing 10 or 25 mg. per cubic centimeter. The gonadotropic hormones were in aqueous solution, and consisted of 2 chorionic gonadotropins, namely follutein (E. R. Squibb & Sons) and A.P.L. (Ayerst, McKenna & Harrison); FSH, a hypophysial follicle stimulating extract made by Dr. H. Jensen of E. R. Squibb & Sons; S.A.P.E., a crude preparation made in this laboratory from dessicated sheep anterior lobe tissue by a method modified from that of Wallen-Lawrence and Van Dyke (7) and gonadin (Cutter Laboratories), a gonadotropic hormone obtained from blood of pregnant mares. The dosages listed in table 2 were computed from various standrads employed for each of these preparations by their originators.

In all experiments the hormones were injected subcutaneously. The rats in the first series of experiments received 3 injections daily for 2 days, and were killed on the fifth day (120 hours) from the time of the first injection.

In the second group of experiments the animals were given 3 daily injections of testosterone propionate (0.1 cc. of the oil solution in each case) for 2 days. The controls received a similar amount of plain sesame oil (table 2). On the third and fourth days 3 injections daily (individual amounts varying from 0.1 to 0.25 cc.) of the various gonadotropins were administered. The rats were killed on the seventh day, that is,

Received for publication August 12, 1940.

[1] This study was supported in part by the Rockefeller Fluid Research Fund of Stanford University School of Medicine. Due acknowledgment is made to Dr. R. MacBrayer and the Ciba Pharmaceutical Products Inc. for a generous supply of testosterone propionate (perandren); to Dr. H. Jensen and E. R. Squibb & Sons for follutein and FSH powder; to Ayerst, McKenna & Harrison for A.P.L.; to Mr. Donald Wonder and the Cutter Laboratories for gonadin. My thanks also are due to Mr. Fred Wong and Mr. Pierre Lassègues for their technical assistance.

120 hours from the time the first injection of gonadotropic hormone was administered.

Ascending doses of testosterone. A series of 4 groups of immature rats were given a total of 6, 15, 30 and 45 mg. of testosterone propionate respectively. As stated before, these consisted of 6 injections administered in the course of 2 days, and the animals were killed in 120 hours from the time of the first injection. In each instance the vaginal introitus was established, and there was an increase in the weights of the uteri which was slightly greater as the dosage was increased (table 1).

A gonadotropic effect was demonstrated histologically. The ovaries contained a

TABLE 1. EFFECT ON UTERINE AND OVARIAN WEIGHTS OF ASCENDING DOSES OF TESTOSTERONE PROPIONATE IN IMMATURE FEMALE RATS

Dosage, test. prop.	No. of rats	Av. body wt.	Av. uterine wt.	Av. ovarian wt.
mg.		gm.	mg.	mg.
6	8	32	0.097	0.010
15	7	35	0.093	0.012
30	7	38	0.123	0.010
45	7	38	0.128	0.012

few large follicles undergoing atresia and an occasional corpus luteum also was seen. On the other hand, there was no appreciable difference in the weights of the ovaries even when the initial dose of 6 mg. was increased to 45 mg. (table 1).

Augmentative Effect of Testosterone

The effect of administering testosterone propionate prior to the injection of various gonadotropic factors is seen in table 2. In all instances there was a definite increase in the weights of the uteri, but the most interesting finding was in regard to the resultant ovarian weight.

The most striking results were obtained when the chorionic gonadotropins were employed. In each case the preliminary injection of 6 or 15 mg. of testosterone pro-

TABLE 2. EFFECT OF UTERINE AND OVARIAN WEIGHTS OF INJECTIONS OF TESTERONE PROPIONATE FOLLOWED BY THE ADMINISTRATION OF VARIOUS GONADOTROPIC HORMONES

Preliminary injections	Gonadotropin	No. of rats	Av. body wt.	Av. uterine wt.	Av. ovarian wt.
			gm.	mg.	mg.
Sesame oil 0.6 cc.	Follutein 1,050 I.U.	6	51	0.122	0.038
Test. prop. 15.0 mg.	Follutein 1,050 I.U.	6	57	0.201	0.089
Sesame oil 0.6 cc.	A.P.L. 750 I.U.	5	35	0.114	0.035
Test. prop. 6.0 mg.	A.P.L. 750 I.U.	5	37	0.148	0.067
Sesame oil 0.6 cc.	FSH 60 U	9	40	0.032	0.020
Test. prop. 6.0 mg.	FSH 60 U	10	38	0.100	0.035
Sesame oil 0.6 cc.	FSH 90 U	6	51	0.050	0.034
Test. prop. 6.0 mg.	FSH 90 U	7	58	0.149	0.039
Sesame oil 0.6 cc.	S.A.P.E. 1.5 cc.	6	37	0.040	0.011
Test. prop. 6.0 mg.	S.A.P.E. 1.5 cc.	6	40	0.136	0.032
Sesame oil 0.6 cc.	Gonadin 60 U	8	36	0.136	0.124
Test. prop. 6.0 mg.	Gonadin 60 U	8	36	0.187	0.113
Sesame oil 0.6 cc.	Gonadin 120 U	8	39	0.187	0.170
Test. prop. 6.0 mg.	Gonadin 120 U	9	36	0.205	0.217

pionate augmented the gonadotropic effect to the extent that the ovaries weighed more than twice as much as those of the controls. The microscopic examination of the ovaries failed to show any noticeable difference in their histologic structure, but an outstanding change was noted in the appearance of the vaginal epithelium. The vaginas of the control group, which received sesame oil as preliminary injections, showed marked cornification. In the animals given testosterone the vaginal mucosa presented an exaggerated picture of mucification although their ovaries were twice as large as those of the control series. This effect bears out the statement of other investigators that testosterone acts directly on the vagina, and apparently its influence in this experiment was great enough to overcome the action of the animals' own gonads.

The results with anterior lobe and equine gonadotropins are somewhat conflicting. In table 2 it it seen that the preliminary administration of testosterone gave augmentation in one instance each with FSH, S.A.P.E. and gonadin, but on the other hand this was not apparent in one experiment with FSH and one with gonadin.

It has been shown that testosterone does not have any gonadotropic effect in hypophysectomized rats (2, 6, 8), and for that reason it is believed that it brings this effect about indirectly by stimulating the anterior hypophysis. This experiment may be interpreted as lending further support to this view. In hypophysectomized rats the chorionic hormone induces merely an hypertrophy of the interstitial tissue of the ovary. The follicle growth and corpus luteum formation obtained in intact animals are considered as resulting from a combined action of this hormone and the anterior lobe gonadotropin normally present. It is logical, therefore, to assume that if injections of testosterone pripionate produce an increased activity of the anterior hypophysis the subsequent administration of chorionic gonadotropin should result in an augmented response.

SUMMARY

The observation of other investigators that testosterone propionate has a gonadotropic effect has been confirmed. It induces the formation of a few large follicles and an occasional corpus luteum in the ovaries of immature rats. On the other hand, it has but little influence on ovarian weight, since the administration of as much as 45 mg. failed to increase the weight above normal in 5-day experiments.

The administration of testosterone propionate prior to the injection of chorionic gonadotropin greatly augments the effect of the latter on ovarian weight. This effect, however, is not so apparent when anterior pituitary or equine gonadotropins are employed, and the results obtained in this study are conflicting.

REFERENCES

1. Hohlweg, W.: *Klin. Wchnschr.* 16: 586. 1937.
2. Nathanson, I. T., C. C. Franseen and A. R. Sweeney: *Proc. Soc. Exper. Biol. & Med.* 39: 385. 1938.
3. Starkey, W. F., and J. H. Leathem: *Proc. Soc. Exper. Biol. & Med.* 39: 218. 1938.
4. Salmon, U. J.: *Proc. Soc. Exper. Biol. & Med.* 38: 352. 1938.
5. Salmon, U. J.: *Endocrinology* 23: 779. 1938.
6. Noble, R. L.: *J. Endocrinol.* 1: 184. 1939.
7. Fluhmann, C. F.: *Proc. Soc. Exper. Biol. & Med.* 29: 1193. 1932.
8. Pencharz, R. I.: *Science* 91: 554. 1940.

THE COMPARATIVE ANDROGENIC POTENCY OF TESTOSTERONE, METHYL TESTOSTERONE AND TESTOSTERONE PROPIONATE ADMINISTERED IN PELLET FORM

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THE PROLONGED ACTIVITY and exceptional efficiency of testosterone and its derivatives are striking when administered in pellet form in laboratory (1, 2) and clinical (3, 4) experiments. All the preparations used appeared extremely potent, which suggests that the previously observed differences in androgenic strength may not be present. The mode of administration of the specific sex steroid compounds is known to influence greatly their androgenic action. Injected subcutaneously in oil solution testosterone propionate is by far the most potent, methyl testosterone is somewhat less active, and testosterone is the weakest (5–8). Percutaneously administered, testosterone in certain concentrations is more potent than testosterone propionate (9). There have been no detailed comparative studies of these substances when administered as pellets, and, as will be indicated, it is suggested that the slow uniform absorption from pellets changes the hitherto accepted ratios of activity.

METHODS

Uniformly compressed pellets of testosterone,[1] methyl testosterone[1] and testosterone propionate[1] were prepared in a mold with 36 identical holes by a die containing plungers to match these holes. The crystalline powder in the holes was subjected to a pressure of 6500 lb. per square inch in a hydraulic press.

The three compounds were tested in 48 male hooded rats, which were castrated and implanted with one pellet each when 21 days old. Sixteen of these animals received implantations of testosterone, 16 of methyl testosterone, and 16 of testosterone propionate. Four animals from each of the above 3 groups were killed, 5, 9, 13 and 17 days after implantation respectively.

Similar tests were carried out on a group of 28 large adult hooded rats, implanted with one pellet each 21 days after castration, and killed 28 days after implantation. Ten of these animals received testosterone, 10 testosterone propionate and 8 methyl testosterone.

Non-implanted castrate male rats of proper age served as controls for all the above groups.

In all animals the pellet was implanted in the subcutaneous tissues of the back. The animals were killed by exsanguination, and the prostates and seminal vesicles were separated and dissected free of adherent tissue and immediately weighed on a

Received for publication September 12, 1940.

[1] The three androgens were supplied as pure crystalline powders through the courtesy of Dr. Ernst Oppenheimer of the Ciba Pharmaceutical Products, Inc.

TABLE 1. THE MEANS AND RANGES OF THE SEMINAL VESICLE, PROSTATIC AND BODY WEIGHTS OF THE GROUPS OF IMMATURE MALE RATS THAT WERE CASTRATED AND IMPLANTED WHEN 21 DAYS OLD

No. animals in each group	Killed: days after implantation		Seminal vesicle weight				Prostate weight				Animal weight			
			T.	T.P.	M.T.	Control	T.	T.P.	M.T.	Control	T.	T.P.	M.T.	Control
			mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	gm.	gm.	gm.	gm.
4	5	mean	80	77	66	7	121	111	124	20	64	68	66	52
		range	76–84	76–78	55–78	6–8	106–132	76–129	106–133	13–25	62–65	60–73	61–76	46–58
4	9	mean	126	136	130	5	210	202	186	13	89	85	78	75
		range	104–155	128–148	113–152	4–6	166–257	155–245	157–246	13–14	84–94	74–92	46–77	72–80
4	13	mean	194	165	165	7	281	210	266	20	93	80	88	103
		range	163–216	143–191	134–198	6–9	261–314	184–258	223–290	13–24	82–98	76–83	81–99	96–117
4	17	mean	206	172	205	6	307	259	299	12	114	113	104	126
		range	202–216	161–188	190–230	5–8	283–338	245–273	252–352	9–17	111–125	105–130	98–113	117–142

(T: testosterone, T.P.: testosterone propionate, M.T.: methyl testosterone.)

damped balance. The contents of the seminal vesicles were carefully expressed and discarded before weighing. The pellets were weighed before implantation and after recovery at the termination of the experiment to determine the total amount of substance absorbed.

RESULTS

In table 1 are listed the averages and ranges obtained from each group of 4 immature castrate males for the seminal vesicles and prostates weighed to the nearest milligram, and the body weight to the nearest gram. In table 2 are listed the averages

TABLE 2. THE MEANS AND RANGES OF THE ORIGINAL WEIGHTS OF THE PELLETS, OF THE AMOUNT ABSORBED FROM THE PELLETS, AND THE CALCULATED EFFICIENCY OF THE ANDROGENS IN THE GROUPS OF IMMATURE CASTRATE MALE RATS

No. animals in each group	Killed: days after implantation		Amount absorbed			Pellet weights			Seminal vesicles (calculated efficiency)			Prostate (calculated efficiency)		
			T.	T.P.	M.T.	T.	T.P.	M.T.	T.	T.P.	M.T.	T.	T.P.	M.T
			mg.	mg.	mg.	mg.	mg.	mg.						
4	5	mean	0.6	0.8	0.8	3.1	8.2	4.1	208	141	125	313	235	253
		range	0.3–0.8	0.6–1.1	0.4–1.0	2.7–3.5	7.7–8.6	3.2–4.0						
4	9	mean	1.0	1.5	1.2	2.7	6.9	3.5	142	107	130	235	158	198
		range	0.7–1.2	1.3–1.6	1.1–1.4	2.5–3.0	3.4–8.4	3.3–3.8						
4	13	mean	2.4	1.6	1.9	4.2	5.1	4.4	87	129	99	126	164	159
		range	2.4–2.5	1.4–2.0	1.6–2.3	3.8–5.1	4.3–6.9	3.8–4.8						
4	17	mean	2.9	1.6	2.5	4.5	4.1	4.2	62	95	75	93	143	115
		range	2.6–3.4	1.3–1.7	2.1–2.7	3.7–5.0	3.6–4.8	3.5–4.6						

The calculated efficiency of the androgens was determined from the following formula:

$$\frac{\text{Ave. organ wt. mg.} \times 100}{\text{Ave. animal wt. gm.}}$$
$$\text{Ave. am't. androgen absorbed mg.}$$

and the ranges of the weights of the pellets implanted in each of these groups, and the average amount of absorbed androgen in milligrams. As the amounts absorbed from the different substances vary, a comparison of their effectiveness may be obtained by correlating the three variable factors: the weight of the stimulated organ;

the weight of the animal; and the weight of the absorbed androgen. These results are included in table 2.[2]

Table 3 combines all these data under similar headings for the groups of large adult males.

The most potent androgen was testosterone when measured by the weight of the prostate gland of the immature animals (table 1). Only the 9-day group deviated. The methyl testosterone pellets produced larger prostates than the testosterone propionate pellets. The same order of activity exists when the seminal vesicles are used as indicators, except that methyl testosterone was most potent in the 5-day group.

In table 3 it is evident that the prostates of the animals treated with testosterone pellets are the heaviest, and that testosterone propionate pellets caused a slightly

TABLE 3. COMPARATIVE EFFECTIVENESS OF PELLETS OF TESTOSTERONE, TESTOSTERONE PROPIONATE AND METHYL TESTOSTERONE IN LARGE ADULT CASTRATE MALE RATS. (EFFICIENCY CALCULATED FROM FORMULA DESCRIBED IN TABLE 2.)

		Testosterone	Testosterone propionate	Methyl testosterone	Control
No. of animals		10	10	8	5
Wt. of animals, gm.	mean	382	397	354	294
	range	330–435	340–420	308–410	270–320
Wt. of seminal vesicles, mg.	mean	690	714	610	80.4
	range	642–760	584–866	489–708	66–95
Wt. of prostate, mg.	mean	1363	1244	1052	58.0
	range	1053–1980	892–1735	792–1352	57–59
Amt. of androgen, absorbed, mg.	mean	4.7	4.6	4.9	—
	range	4.2–5.6	3.6–5.6	4.4–5.3	—
Original wt. of pellet, mg.	mean	7.6	12.5	8.6	—
	range	6.7–8.3	11.3–13.4	8.1–9.2	—
Efficiency based on seminal vesicles		37	38	35	—
Efficiency based on prostate		75	69	60	—

greater prostatic enlargement than methyl testosterone pellets. The heaviest seminal vesicles were present in the group treated with testosterone propionate pellets; testosterone pellets produced heavier seminal vesicles than methyl testosterone. In these large adult male castrates the differences in weights of the prostates between the testosterone treated group (heaviest) and the methyl testosterone treated group (lightest), seem definite but are not significant when analyzed statistically. The differences between the weights of the seminal vesicles in the three groups are slight and not significant.

Absorption from the implanted pellets which had been uniformly compressed varied with the substances used, the period of implantation, and with the original sizes and weights of the pellets (table 2, 4). Some of the wide variations in the amount of androgen absorbed in the shorter implantation intervals may have resulted from

[2] These results were obtained by dividing the average weight of the stimulated organs in milligrams times 100, by the average weight of the animal in grams, and then dividing this number by the average weight of the absorbed androgen in milligrams.

the possible loss of minute chips or fragments from the recovered pellets. Over the longer implantation intervals less marked variations in the amounts absorbed were noted. The larger pellets of the 3 androgens used in the adult castrate groups (table 3) were selected by weight to obtain almost equivalent absorption over the 28-day interval. This minimized the absorption factor when the extent of androgenic action was determined.

In the 3 groups of immature castrates killed 5 and 9 days respectively after implantation the averages and ranges of the weights of the prostates and of the seminal vesicles are of the same order of magnitude. The smallest amount of androgen absorbed in these two intervals was from the pellets of testosterone, so that in correlating the three factors testosterone is the most efficient, and testosterone propionate the least (table 2). However, these differences are relatively slight.

In the groups killed 13 and 17 days respectively after implantation the least amount of androgen absorbed was from the testosterone propionate pellets, which substance, therefore, becomes the most efficient (table 2), when measured by both the seminal vesicles and prostates.

Each of the 3 androgens was absorbed almost equally in the groups of large adult castrates (table 3), thus altering the discrepancy between organ weight and efficiency as noted in the 13 and 17-day groups of immature castrates. In these large adult castrates testosterone showed the greatest efficiency when measured by the weight of the prostates, and testosterone propionate was most efficient when the seminal vesicles were the indicators (table 3). As determined by both organs the differences between the 3 compounds were not marked.

DISCUSSION

The tendency toward uniformity in androgenic potency of the 3 compounds when administered as pellets stands in sharp contrast to the tremendous differences in potency, 4 to 6-fold between testosterone propionate and testosterone, and approximately 2-fold between methyl testosterone and testosterone, when administered in oil. The similarity is the result of a definite gain in activity of testosterone and methyl testosterone. Testosterone propionate appears to maintain the same degree or only a slightly greater activity when compared to oil injections. These comparisons with observations based on oil injections are mainly approximations, for the differences in animal weights, amounts injected, and durations of injections varied.

The similarity in the degree of activity of these 3 compounds indicates that some of the present conceptions concerning effectiveness of the androgens may require modification. It is held by some (10) that the esterification of testosterone by a fatty acid enhances its activity comparable to that produced by the 'X-substance' of Laqueur, and that the function of both is to impede the physiologic activity which is developed only as the blocking group is hydrolyzed. The relative similarity in action of the 3 compounds when administered as pellets suggests that a uniform and slow absorption from the site of injection may be the essential factor by which esterification enhances activity. This uniform rate of absorption from pellets may permit the concentration of the androgen in the tissues to be maintained at a constant level, whereas with oil injections absorption may be rapid and uneven, producing variations in the concentration of the injected androgen. It has been shown that testosterone propionate (11) and methyl testosterone (12) are inactivated by the liver, and unpublished experiments indicate that testosterone is metabolized similarly, indicating that inactivation is not a factor in the matter of comparative potency.

Our findings coincide with the observations of Deanesly and Parkes (1) that tablets of testosterone and of testosterone propionate were equally effective in producing

the enlargement of the prostates and seminal vesicles in castrate male rats, and with the recent report by Emmens and Parkes (13) that testosterone tablets are to some extent more effective than methyl testosterone tablets. Testosterone in certain concentrations is more effective than testosterone propionate, as shown by observations on percutaneous application (7). This also indicates that the method of administration plays an important role in classifying the relative activity of these androgens.

If the efficiency of testosterone and of testosterone propionate were contrasted on an equimolecular basis the latter would show a greater comparative activity because only a portion of the absorbed molecule is physiologically active androgen.

SUMMARY

Pellets of testosterone propionate, testosterone and methyl testosterone were implanted in groups of immature and adult castrate rats. The immature animals were castrated and implanted with a pellet when 21 days old, groups were killed 5, 9, 13 and 17 days later. The adult animals were implanted 3 weeks after castration, and killed 4 weeks after implantation. To permit closer analysis, the efficiency of the compounds was determined by correlating the weight of the stimulated organ, the weight of the animal, and the weight of the absorbed androgen.

Testosterone propionate pellets were most efficient when measured by the seminal vesicles and prostates of the 13 and 17-day immature groups, and the seminal vesicles of the adult castrate group. Testosterone pellets were most efficient in the 9 and 13-day immature groups and the prostates of the adult castrate group. Testosterone pellets generally produced the largest seminal vesicles and prostate in all groups, and permitted the greatest absorption.

The 3 compounds administered as pellets showed a surprising uniformity of androgenic potency when contrasted with the marked differences that occur when these compounds were administered in oil solution. It is assumed that the uniform and slow absorption from pellets permits testosterone and methyl testosterone to approach testosterone propionate in activity, and indicates that this may be the important effect produced by the esterification of the latter.

The technical assistance of Mrs. R. Helmuth is gratefully acknowledged.

REFERENCES

1. Deanesly, R., and A. S. Parkes: *Proc. Roy. Soc. London, s. B.* 124: 279. 1937.
2. Deanesly, R.: *Proc. Internat. Coll. Singer-Polignac* 1: 23. 1938.
3. Vest, S. A., and J. E. Howard: *J.A.M.A.* 113: 1869. 1939.
4. Biskind, G. R., R. F. Escamilla and H. Lisser: *J. Clin. Endocrinol.* 1: 38. 1941.
5. Deanesly, R., and A. S. Parkes: *Biochem. J.* 30: 291. 1936.
6. Moore, C. R., and D. Price: *Anat. Rec.* 71: 59. 1938.
7. Miescher, K., A. Wettstein and E. Tschopp: *Biochem. J.* 30: 1970. 1936.
8. Korenchevsky, V., M. Dennison and M. Eldridge: *Biochem. J.* 31: 475. 1937.
9. Nelson, D., R. R. Greene and J. A. Wells: *Endocrinology* 26: 651. 1940.
10. Elderfield, R. C.: *Cold Spring Harbor Symp. Quant. Biol.* 5: 1. 1937.
11. Biskind, G. R., and J. Mark: *Bull. Johns Hopkins Hosp.* 65: 212. 1939.
12. Biskind, G. R.: *Proc. Soc. Exper. Biol. & Med.* 43: 259. 1940.
13. Emmens, C. W., and A. S. Parkes: *J. Endocrinol.* 1: 323. 1939.

THE EFFECTIVENESS OF ANDROGENS DURING INANITION IN THE CHICK[1]

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Chicks given an amount of food insufficient for normal body growth proved to be more responsive to anterior pituitary hormone and responded to smaller amounts of androgens than did birds whose nutrition was normal (1, 2). These results suggested the possibility of using such animals for bioassay of androgens. It was also observed that gonad weights in the dieted chicks were lighter than in the controls, which indicated that there was a considerable suppression of the secretion of pituitary gonadotropin. A study of animals suffering from inanition, therefore, affords a valuable approach to the problem of pituitary control of testis growth and male hormone secretion in the chick, and would be similar to the study now being made on the rat (3).

The use of chick comb growth with its possible application to male hormone assay has been investigated by others (4, 5), but the variability in response seemed to make this test impracticable. Chicks placed on limited diet also proved to be variable but the growth of the comb was proportionately much greater than in the normal birds on the same hormone treatment. The use of dieted chicks thus promised to eliminate some of the difficulty encountered in the normal birds. This paper presents an analysis of the effect of subcutaneous administration of androgens in relatively small dosages into limited diet animals. The limitation of diet was more severe than in previous experiments and the number of animals was somewhat more extensive; 536 chicks were used in this work.

MATERIALS AND METHODS

Single comb White Leghorn chicks were employed in all experiments. The birds were reared in a battery brooder and placed on a starting mash when 2 days old. Chicks were permitted to eat all they desired when food was present, but food was removed on alternate days following the first 24-hour feeding period. It was found necessary, however, to remove the food for increasingly longer periods of time as the animals became older, and although no detailed analysis is made in this paper of the total amount of food consumed, it may be noted that the weight of the animals increased from an average of 40 gm. on the second day to about 65 gm. on the fifteenth day. The chicks were without food for 24 hours before killing but in a few instances in which there was food in the crop, this organ was removed before the animal was weighed. All body weights, therefore, represent body weight less crop content. Combs were removed by cutting with scissors parallel to the head and were weighed in all experiments in order to secure the most accurate possible determination of comb size. Comb averages are presented in the tables by both actual weight and as a per-

Received for publication July 16, 1940.

[1] Contribution No. 87 of the Waterman Institute and No. 287 of the Zoology Department of Indiana University.

[2] The author is indebted to the Schering Corporation of Bloomfield, New Jersey, for the crystalline androgenic preparations used in this study.

[3] This work was supported in part by a grant from the Difco Laboratories Inc. of Detroit, Michigan, as a portion of their program for research on the internal secretions.

centage of body weight. The use of a factor for comb weight calculated as a percentage of body weight will be considered in the discussion of the data.

Four androgens were injected during this study: oreton (testosterone propionate), oreton-B (dihydroandrosterone benzoate), a testicular male hormone preparation made by the Difco laboratories, and androstenedione in one series. The hormones were all administered subcutaneously in 0.1 cc. solutions of sesame oil.

RESULTS AND DISCUSSION

The best method for representing comb averages presented a major problem. Inspection of the data indicated that there was a correlation between body and comb weights in many of the animals, and that it might be advantageous to calculate the combs as a percentage of body weight. This, or a similar correction, appeared to be essential whenever comparisons were to be made with normal animals or between

TABLE 1. A COMPARISON OF BODY AND COMB WEIGHTS OF NORMAL AND LIMITED DIET CHICKS, AT 10 AND 15 DAYS OF AGE

Animals	No.	Av. body wt.	Av. comb wt.	CV%	Comb-body %	CV %
10-day limited diet	27	45.0	12.2±2.04	16.0	.0278± .0044	15.8
10-day normal diet	15	61.6	22.5±6.5	28.9	.0377± .0244	64.6
15-day limited diet	46	59.8	16.7±8.8	52.6	.0279± .0115	41.2
15-day normal diet	18	106.0	115.0±88.2	76.9	.1010± .0686	67.9

series in which there was any considerable difference in mean body weights. It is necessary to determine the validity of such a correction factor, and this was done by comparing representative series of both normal and limited diet control chicks of 10 and 15 days of age. The mean and coefficient of variation for comb weight and for comb size as a percentage of body weight was computed and these data appear in table 1.

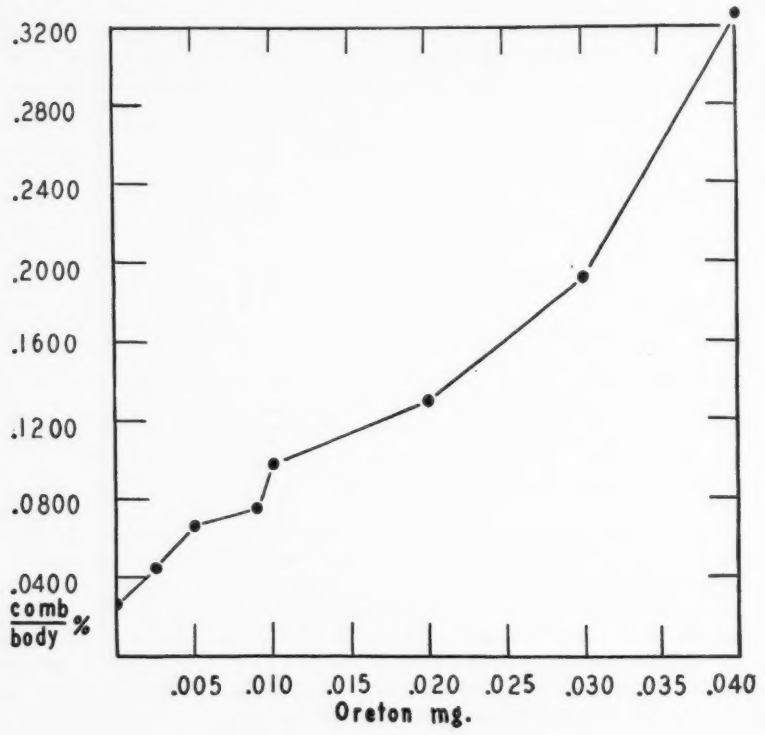
The results are in contrast to those of previous work in which limited diet chicks appeared to be as variable as the normal birds. The data in table 1 are based on a greater number of animals and with more severe fasting than in earlier experiments, and these results indicate that there was actually less variation in the limited diet animals. The coefficient of variability in the dieted birds at 10 days was 16% for the comb weight and 15.8% for the comb/body weight percentage. The corresponding figures for the normal animals were 28.9% and 64.6%. At 10 days there would appear to be no advantage in the use of the percentage weight determination of comb size since there was no significant difference in variability in the limited diet chick, whereas the normal diet animals were even more variable. At 15 days, however, both the limited and normal diet birds showed more variation in comb weights; but when the comb average on the basis of the body weight correction was used, this figure proved to be less variable in both series and the inanition chicks again had a lower coefficient of variation. Since most of the series reported are 15-day limited diet ex-

TABLE 2. 10-DAY LIMITED DIET CHICKS GIVEN ORETON-B FROM THIRD TO NINTH DAYS INCLUSIVE

Treatment	No.	Body wt.	Comb wt.	Comb/Body wt.%
Control	12	41.8	13.5	.0323
0.005 mg. oreton-B daily	10	41.8	16.2	.0388
0.010 mg. oreton-B daily	10	44.8	22.1	.0494
0.020 mg. oreton-B daily	10	44.3	26.6	.0601

periments, it was decided to employ the body weight corrections and to use the resultant factors for comparison of the results.

Although it was noted previously (2, 5) that the shorter time periods of injection were less efficient in promoting comb growth, a few chicks were treated over the period from the third to ninth day after hatching and were killed on the tenth day. The results of these experiments are summarized in table 2 for comparison with longer series. The relatively small number of animals is offset to some degree by the uniformity of the results because as was noted in table 1, the variability at 10 days



RESPONSE OF CHICK COMB TO ORETON

was not extensive. Control combs had an average weight of 13.5 mg. and this comb average was increased to 26.6 mg. by the daily injection of 0.020 mg. of oreton-B. The comb size in the series given 0.005 mg. of the hormone daily was 2.7 mg. greater than that of the controls, and if this is used as a standard for comparison the expected comb weight in the 0.020 mg. treated series should be 24.3 mg. Since the actual weight was 26.6 mg., the calculated result, therefore, was approximately 85% of the observed. These data are even more significant when similar comparison is made of the comb/body weight factors. Treatment with 0.005 mg. of the androgen produced an increase of .0065 in comb-body weight ratio, and the injection of 0.020 mg. increased this ratio to .0601 or a net increase of .0278. The calculated comb ratio, using the 0.005 mg. series as a base, should have been .0583 and is, therefore, 97% of the actual

comb/body weight figures. This illustrates a distinct advantage in the use of the body weight correction for the evaluation of these results.

A much more extensive study was made of the longer series in which either oreton or oreton-B was injected from the third to the fourteenth day. Although the amount of hormone injected was less than twice as much as in the shorter time series the net increase in comb sizes for a given amount of androgen was more than doubled. The data for the oreton series are summarized in figure 1 but for the purpose of more detailed analysis the oreton-B injected series are presented in table 3 which also includes the data for the testis androgen and the androstenedione series.

The general appearance of the graph is that of a straight line with no evidence of

TABLE 3. INJECTION OF ORETON-B, ANDROSTENEDIONE, AND TESTICULAR ANDROGEN INTO CHICKS FROM THIRD TO FOURTEENTH DAY AFTER HATCHING. KILLED ON FIFTEENTH DAY

Treatment All injections from 3d to 14th day	No.	Body wt.	Comb wt.	$\frac{\text{Comb}}{\text{Body}} \times 100$	Testis wt.	Testis correction
		gm.	gm.		mg.	
Control[1]	46	59.8	16.7	.0279	13.8	.0207
Oreton-B daily						
0.0025 mg.	26	63.0	23.2	.0368	14.6	.0253
0.005 mg.	24	68.7	29.4	.0428	16.6	.0258
0.006 mg.	13	58.4	24.4	.0418	11.9	.0351
0.008 mg.	16	61.3	25.9	.0423	10.6	.0406
0.010 mg.	35	64.0	43.8	.0686	13.0	.0527
0.020 mg.	31	62.5	75.0	.1200	11.1	.1090
0.030 mg.	18	70.2	114.9	.1676	12.6	.1300
0.040 mg.	20	65.0	152.7	.2350	12.1	.1940
0.050 mg.	16	74.6	270.5	.3630	15.5	.2340
Androstenedione daily						
0.010 mg.	14	65.2	22.9	.0351	13.9	.0253
Testis androgen capon units (C.U.) daily						
0.010 C.U.	13	72.0	22.5	.0313	—	—
0.020 C.U.	12	71.0	22.8	.0320	—	—
0.100 C.U.	13	71.9	26.5	.0369	—	—

[1] Same as table 1.

a plateau at the higher dosages, and it may be concluded that any plateau would have to occur following administration of larger amounts of the hormone. It is of special interest to note that very significant comb growth was produced by the injection of 0.0025 mg. daily of oreton. This was a total of 0.030 mg. of the testosterone propionate, and, of course, represents a smaller amount of the free testosterone. Work with normal diet chicks showed that oreton (testosterone propionate) was not as effective as the oreton-B (dihydroandrosterone benzoate) in promoting comb growth (6, 7). This observation does not apply in the case of the limited diet animals, and its significance will be discussed later. Attention should also be called to the fact that the comb response at the 0.0025 mg. and 0.005 mg. dosage levels is quite marked and without any indication of lag or loss of effectiveness of the hormone through a necessity for priming the comb. The efficacy of the oreton is apparently sufficient to overcome the priming effect with dosages as low as 0.0025 mg. which also is in contrast with results in the oreton-B series. The total number of animals represented in

the graph of the oreton series is 137 and the smallest number of any one dosage is 11 at 0.0025 mg. daily. The total number of animals should of course be supplemented for further study, especially at the critical extremes of the dosages. The oreton-B data are somewhat more satisfactory in this respect since there were 199 chicks used and these also included experiments with 0.006 mg. and 0.050 mg. daily which allows additional comparison.

There was a definite plateau in the comb response to smaller dosages as was noted above. The amount of comb growth elicited by 0.0025 mg. of oreton-B was significant but almost the same increment was produced by injection of 0.006 mg. and 0.008 mg. of oreton-B as that which occurred at the 0.005 mg. level. With an increase to 0.010 mg. of the hormone daily, however, the comb showed a very marked growth and at the 0.020 mg. and 0.040 mg. dosages, the size increase was remarkably uniform. The great increase in the comb averages following the administration of the 0.050 mg. of the hormone was puzzling at first and even more inexplicable than the plateau which occurs at the lower dosages. A consideration of the factors which control comb growth in the chick suggested a possible interpretation for this variation and a further correction which might be made for comb determinations.

It has been demonstrated (8, 9) that two factors operate in comb growth; the first of these is strictly "non-hormonal" and is the increase in the comb which is proportional to body growth. This is illustrated by the comb growth which may take place in the capon. The second factor promoting comb growth is the amount of androgenic substance which is present and which will produce an increment in excess of proportional body growth. This "hormonal" growth may be produced either by the secretion of male hormone from the animal's own gonads or by the injection of some comb growth promoting substance. It was demonstrated (6, 9) that both the non-hormonal and hormonal effects are present early in the White Leghorn chick and there was evidently some androgen secreted by the chick testes at 10 and 15 days of age. It was concluded in the light of these observations that if average testicular weights differed in the various series, those groups with heavier gonads would secrete more androgen than would those with lighter gonads. Comb averages should, therefore, be corrected for uniform testis weight, as well as for body weight. Correction would thus be made for both the non-hormonal and hormonal variables for comb growth and such corrections would, if valid, leave the amount of injected hormone as the only major variable.

The factors which resulted from correcting the mean comb weights in the oreton-B experiments by adjusting all the series to a uniform testis weight of 10 mg. are summarized in table 3. Much of the difficulty which was encountered in trying to interpret the effects of the lower dosages was overcome following this correction, especially the discrepancy at the 0.006 mg. and 0.008 mg. levels. Considerable lag in comb response was still evident which was further emphasized by the fact that at the 0.010 mg. dosage there was a very marked increase in average weight. Much of the hormone injected at these lower dosages must have been used to prime the comb and have been close to the threshold level.

Perhaps the most interesting results of the application of the testis correction to the data are found at the higher dosages of the hormone, where the priming effect is relatively less important. The comb response to a given dosage may be forecast with surprising accuracy as a summation of the reactions to lower dosages. The only hormone level at which there is a very marked divergence is in the 0.03 mg. series, the remaining series correspond closely to the expected size. Some of the possible calculations are presented in table 4 in order to illustrate this point.

These results are noteworthy particularly in the 0.050 mg. series. When body

weight correction only was made the data for this group of birds was not typical of the rest of the upper range, but the response when corrected for testes weight differs from the calculated weight by only 2% and 5%. The testes, therefore, do exert considerable influence on the comb response of the limited diet chicks at this age.

The complexity of the physiological relationship between pituitary and testes in the limited diet birds can be best illustrated by comparing the data for these chicks with similar experiments in the normal. Effectiveness of oreton-B on comb growth in the normal exceeded that of oreton and this phenomenon was interpreted as a difference in the interaction of oreton and oreton-B with some testicular androgen. This conclusion was further supported by results of administering mixtures of various androgenic preparations (7) in which oreton-B mixtures were more efficacious. The possibility exists that during inanition there is either a complete cessation of male hormone secretion in the chick or that there may be a marked difference in the utilization of the hormone. The results in the oreton-B limited diet series seem to show

TABLE 4. COMPARISON OF ACTUAL COMB GROWTH WITH THAT INDICATED BY SUMMATION OF THE RESPONSES TO LOWER DOSAGES

Treatment	Summation	Summation weight	Actual weight	%
0.020 mg.	2×0.010 mg.	1054	1090	96.7
0.030 mg.	3×0.010 mg.	1581	1300	121.5
	0.020+0.010 mg.	1617	1300	128.5
0.040 mg.	2×0.020 mg.	2018	1940	104.0
	0.030 mg.+0.010 mg.	1827	1940	94.2
0.050 mg.	0.020 mg.+0.030 mg.	2390	2340	102.0
	0.010 mg.+0.040 mg.	2467	2340	105.0

definitely that male hormone is secreted in these chicks, but a comparison with the normal animals in table 1 shows that this must be small in amount and in most cases undoubtedly of sub-threshold level because comb size remains constant between 10 and 15 days. The results do indicate, on the other hand, that there is a more efficient utilization of the hormone as evidenced by the greater responsiveness of the limited diet animals. This might be due to differences in the rate of oxidation, of excretion, or effect on the end organ. Additional study on these problems is needed.

Four series were used to test the practicability of the limited diet animals as a test for other androgenic substances. Androstenedione injected in 0.010 mg. amounts daily produced combs which were equivalent in size to those in the 0.0025 mg. oreton-B series. This was true for both body weight corrected data and the testes weight factor, and the results were sufficiently higher than the control to demonstrate the androgenic activity of the hormone in this test method. The testis preparation employed was standardized in capon units and the dosages are represented as fractions of those units. It is again evident that the priming of the comb requires a considerable portion of the available androgen at the dosage levels employed, but it may be noted that the androgenic effect on the comb of the 0.100 C.U. of testis androgen is slightly greater than that produced by 0.010 mg. of androstenedione, and is equal to that produced by 0.0025 mg. of the oreton-B. It is unfortunate that in these particular series the gonad weights were not taken and the testis correction could not be made.

These results show that it is feasible to use limited diet chicks for testing the effect of small dosages of androgens, but at lower dosages the combs should be first stimulated by priming with male hormone. Unpublished data have demonstrated

that it is possible to secure a positive comb response by injecting as little as 0.001 mg. of testosterone propionate daily for 10 days providing the combs have been primed before the test began. Dihydroandrosterone benzoate will also produce significant increases in comb averages with a total dosage of 0.025 mg. which would be 0.0375 capon units of this hormone.

The physiological problems which are encountered in these experiments are significant. It was shown (2) that limitation of food served to inhibit the secretion of gonadotropic hormone from the pituitary and it was thought that there was a complete cessation of secretion. More extensive study, as in the oreton-B series, shows that the inhibition of the pituitary secretion was not completely stopped but is variable. This is evidenced by the 15.5 mg. testis average in the chicks treated with 0.050 mg. of hormone and the 10.6 mg. testis average in the 0.008 group and illustrates that the maximum dosages do not necessarily produce the maximum inhibition. Correction for testes weight in the comb averages implies a direct correlation between weight of testes and amount of androgen secreted. This is probably not absolutely a valid assumption as it seems logical to expect that after a certain amount of inhibition the testis will cease entirely to secrete androgen and, therefore, a correction for gonads of very low weights would be misleading to some extent.

The gonadotropic content of the pituitaries in various limited diet series is being tested and a cytological study of pituitaries from similar experiments is in progress. The latter work is being done by Dr. Fernandus Payne of this laboratory. The implications of androgen secretion and the interaction of the injected and testicular hormones is being subjected to further test by comparison of the cockerels with limited diet pullets of corresponding ages which received the same treatment. It is planned to present this material soon and to include experiments with priming dosages.

SUMMARY

Single comb White Leghorn chicks placed on a limited supply of food were less variable in their comb growth than were the animals on a normal diet. When the birds were 15 days of age, the factor of comb size as a percentage of body weight (comb weight/body weight $\times$ 100) proved to be less variable than average comb weight.

Experiments with oreton (testosterone propionate), oreton-B (dihydroandrosterone benzoate), androstenedione, and a testicular androgen preparation, produced significant comb growth. Injections from the third to fourteenth day were relatively much more efficient in stimulating comb increment than injections which were made from the third to ninth day only. Oreton was more efficacious than oreton-B; this is contrary to previous work on normal diet birds injected with these hormones. At lower dosage levels of 0.0025 mg., 0.005 mg. daily injection of androgen, there was evidence that a considerable portion of the androgen was necessary for initiation of comb growth (priming effect). An additional correction of the comb factor by adjusting the averages for uniform testes weights proved to be practical in the oreton-B series and it was possible to forecast within 2 to 5% the actual comb growth at certain dosages of the hormone.

REFERENCES

1. Byerly, T. C., and W. H. Burrows: *Endocrinology* 22: 366. 1938.
2. Breneman, W. R.: *Endocrinology* 26: 1091. 1940.
3. Mulinos, M. G., and L. Pomerantz: *J. Nutrition* 19: 493. 1940.
4. Burrows, W. H., T. C. Byerly and E. I. Evans: *Proc. Soc. Exper. Biol & Med.* 35: 50. 1936.
5. Frank, R. T., E. Klempner and F. Hollander: *Proc. Soc. Exper. Biol. & Med.* 38: 853. 1938.
6. Breneman, W. R.: *Endocrinology* 23: 44. 1938.
7. Breneman, W. R. *Endocrinology* 24: 55. 1939.
8. Hardesty, M.: *Am. J. Anat.* 47: 277. 1931.
9. Breneman, W. R.: *Indiana University Science Series* 7: 5. 1938.

THE GROWTH STIMULATING EFFECT OF SMALL DOSES OF TESTOSTERONE PROPIONATE IN THE CASTRATE ALBINO RAT

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IT IS WELL ESTABLISHED that the sex function influences the growth of the animal. Thus, male rats castrated in early life tend to remain shorter than normal (1–6). It appears from this that sex hormones could possibly serve as stimulators of body growth. Yet when this hypothesis was first tested it was found that testosterone propionate when chronically administered to the albino rat instead of stimulating, actually depressed the growth of the animal (7). This effect was considered to be due to a depression of the pituitary growth function as a result of overdosage with sex hormones (8–16).

It therefore became interesting to determine whether smaller doses of this hormone would prove less inhibiting or possibly stimulating to the body growth processes of the organism.

In a previous communication (17) it was shown that 50γ of testosterone propionate administered daily (except Sunday) from 26 to 80 days did stimulate the growth of normal male albino rats.

TABLE 1. COMPARISON OF INITIAL AND FINAL BODY WEIGHTS IN GRAMS OF CASTRATED RATS TREATED WITH 50γ OF TESTOSTERONE PROPIONATE DAILY (EXCEPT SUNDAY) WITH THEIR RESPECTIVE CONTROL FINDINGS

	Initial wt. 26 days	Final wt. 80 days	Gain
Untreated	49.5±2.1	166.8±4.4	117.2±4.1
Treated	47.3±1.2	180.9±2.8	133.3±3.3
Difference	2.2±2.4	14.1±5.2	16.1±5.3
S.R.	0.92	2.7	3.02

In this study the effect of this same dosage was observed in a group of castrated animals. For this purpose 30 male albino rats of Wistar Institute strain were castrated at 22 days of age after which they were divided into 2 groups. *Group 1* contained 15 animals each of which received daily intraperitoneal injections of 50γ testosterone propionate (perandren) from 26 to 80 days. *Group 2*, also consisting of 15 castrates remained as uninjected controls. All animals were otherwise similarly treated, Purina Dog Chow and green vegetables being used as a diet. Water was constantly present. Twenty-four hours after the last injection all animals were anesthetized with ether and killed by sectioning of the carotids.

Weekly, initial and final body weights were taken and all data were subjected to statistical analysis, the criterion for probable significance being a "significance ratio" of 3 or more (18).

Received for publication July 10, 1940.

RESULTS

From table 1 it may be seen that no significant difference existed between the initial body weights of the 2 castrated groups. However, the final weight of the treated castrates (180.9 ± 2.8 gm.) was 14.1 ± 5.2 gm. heavier than the final weight of the untreated castrates (166.8 ± 4.4 gm.). The significance ratio of 2.7 for this difference falls just short of the criterion set by us for probable significance. However,

TABLE 2. COMPARING INITIAL AND FINAL BODY WEIGHTS, ACTUAL AND PERCENT GAINS IN BODY WEIGHTS OF NORMAL UNTREATED, NORMAL TREATED, CASTRATE UNTREATED AND CASTRATED TREATED ANIMALS OF SIMILAR STRAIN

	Initial wt. 26 days	Final wt. 80 days	Gain	% gain
Normal untreated (12 animals)	45.7	174.1	128.4	281
Normal treated (12 animals)	45.2	193.8	148.6	328
Castrate untreated (15 animals)	49.5	166.8	117.3	236
Castrate treated (12 animals)	47.3	180.9	133.6	282

a comparison of gains in body weight between the treated (133.3 ± 3.3 gm.) and untreated (117.2 ± 4.1) castrates discloses a difference of 16.1 ± 5.3 gm. in favor of the treated animals. The significance ratio of 3.02 for this difference does indicate a probable significance.

DISCUSSION

From these results it is seen that castrate animals treated with 50γ testosterone propionate grow in excess of the non-treated castrate. Since it has previously been shown that castration inhibits growth in the male albino rat it appears that the dosage used tends to counteract such castration-determined growth depression. This can be more readily appreciated from table 2 which utilizes data reported here and elsewhere (17) to compare percentage gains in body weight of differently treated groups of animals of the same strain. Figure 1 shows this difference of growth tendency. From these it may be seen that while the castrate rat during the period of observation gained 45% less in body weight, the normal treated rat gained 47% in excess of the normal untreated animals. The percentage gain of the treated castrate was practically identical with that of the normal (282% and 281% respectively).

That growth changes observed after testicular ablation or sex hormonal administration represent true body growth effects may be gathered from other studies (6, 7, 13).

The exact reasons for the growth stimulating effect of testosterone propionate are unknown. It seems, however, that this hormone possesses inherent growth stimulating qualities. At this time it may be considered to stimulate specifically the growth of the accessory sex organs which have a selective receptivity for this particular hormone. In a less specific manner other tissues may respond by added growth provided that the hormone concentration is insufficient to inhibit the more generalized growth function of the pituitary gland. Finally, it is possible that small doses of testosterone propionate may have a direct stimulating effect upon the growth function of the pituitary. So far no significant weight changes have been observed in the pituitary glands of animals so treated. It is hoped however that cytological studies now in progress will throw light on this question.

SUMMARY

Testosterone propionate administered intraperitoneally daily (except Sunday) in 50-gamma doses to castrated male albino rats from 26 to 80 days of age led to a "prob-

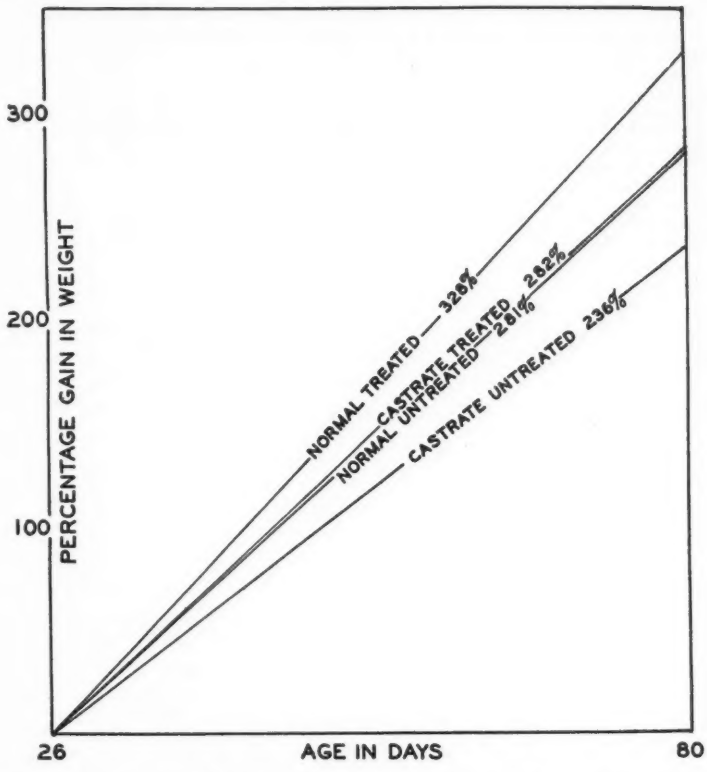

Fig. 1. PERCENTAGE GAINS IN WEIGHT OF NORMAL TREATED, NORMAL UNTREATED, CASTRATE TREATED AND CASTRATE UNTREATED MALE ALBINO RATS FROM 26 TO 80 DAYS OF AGE. Castration at 22 days of age. Treatment consisted of daily intraperitoneal injections of 50γ testosterone propionate.

ably significant" increase in growth as compared to untreated castrates. Comparing the data herein presented with previously reported observations of animals of similar strain it was found that castrated albino rats treated with this dosage showed a total percentage gain in weight which was practically identical with that shown by the non-treated normally growing rat.

The possible reasons for the growth effect of testosterone propionate are considered and it is felt that these may depend upon an inherent growth stimulating quality which strikes specifically at the accessory sex organs and more generally at other somatic tissues. There is a possibility that this hormone in small dosage may stimulate the growth function of the pituitary gland.

The authors gratefully acknowledge the cooperation of the Ciba Pharmaceutical Products Company Inc., for partially defraying the expenses and supplying the perandren used in this study.

REFERENCES

1. DONALDSON, H. H., AND S. HATAI: *J. Comp. Neurol.* 21: 155. 1911.
2. EVANS, H. H., AND M. E. SIMPSON: *Anat. Rec.* 35: 36. 1927.
3. KORENCHEVSKY, V.: *J. Path. & Bacteriol.* 33: 607. 1930.
4. COMMINS, W. D.: *J. Exper. Zool.* 63: 573. 1932.
5. LAWLESS, J. J.: *Anat. Rec.* 66: 455. 1936.

6. Rubinstein, H. S., A. R. Abarbanel and A. A. Kurland: *Endocrinology* 25: 397. 1939.
7. Rubinstein, H. S., A. A. Kurland and M. Goodwin: *Endocrinology* 25: 724. 1939.
8. Steinach, E., and G. Holzknecht: *Arch. f. Entwicklungsmech. d. Organs* 41: 490. 1917.
9. Bugbee, E. P., and A. E. Simmond: *Endocrinology* 10: 360. 1926.
10. Spencer, J., F. E. D'Amour and R. G. Gustavson: *Endocrinology* 16: 647. 1932.
11. Spencer, J., R. G. Gustavson F. E. D'Amour: *Proc. Soc. Exper. Biol. & Med.* 28: 500. 1931.
12. Spencer, J., F. E. D'Amour and R. G. Gustavson: *Am. J. Anat.* 50: 129. 1932.
13. Zondek, B.: *Lancet.* 230: 10. 1936.
14. Zondek, B.: *Lancet* 231: 842. 1936.
15. McCullagh, D. R., and E. L. Walsh: *Endocrinology* 19: 466. 1935.
16. Hamilton, J. B., and J. M. Wolfe: *Endocrinology* 22: 360. 1938.
17. Rubinstein, H. S., and M. L. Solomon: *Proc. Soc. Exper. Biol. & Med.* (in press).
18. Pearl, R.: Medical Biometry and Statistics. Saunders, Philadelphia, 1930.

THE PHENOMENON OF ANTAGONISM BY PITUITARY EXTRACT IN THE ABSENCE OF THE THYROID GLAND[1]

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SINCE THE DEMONSTRATION by Smith (1) that crude extracts of the anterior hypophysis, when injected intraperitoneally, inhibit the gonadotropic action of pituitary implants, it has been shown (2, 3) that purified pituitary extracts exhibit this action against the gonadotropins of urine, pregnant mares' serum and of the pituitary gland.

Efforts have been made to establish the antagonistic principle as a separate entity. Evans et al. (2) reported that they were able to prepare an 'antagonist' substantially free of gonadotropic, thyrotropic, adrenotropic, and lactogenic factors. More recently however, a report from the same laboratory (4) identifies the antagonistic action as a property of both the follicle-stimulating and luteinizing hormones, especially the former. The claim of Bunde and Hellbaum (5) that the antagonistic principle shows a heat stability not possessed by the gonadotropic activity of the same extracts has been denied by Bischoff (6) and by Jensen and Tolksdorf (7).

Several attempts have been made to identify the antagonistic principle with other factors present in hypophyseal extracts. Recently Tolksdorf and Jensen (8) have suggested that the factor which inhibits gonadotropic action is identical with the thyrotropic hormone. They point out that efforts to separate the antagonistic and thyrotropic principles have been unsuccessful and emphasize the earlier finding (9, 10, 11) that gonadotropic action is inhibited by experimentally produced hyperthyroidism. Tolksdorf and Jensen reason that intraperitoneal injection of thyrotropic hormone stimulates the thyroid to secrete excessive amounts of its hormone, which in turn inhibits (antagonizes) the effect of simultaneous subcutaneous treatment with gonadotropin.

Since the 'antagonist' is most effective when administered intraperitoneally,[2] it becomes necessary to assume that the thyrotropic principle is also much more effective when administered by the intraperitoneal than by the subcutaneous route. Of equal importance in the hypothesis of Tolksdorf and Jensen, and recognized by them, is the assumption that the phenomenon of antagonism cannot be produced in the absence of the thyroid gland. However, Evans et al. (2) had earlier demonstrated the effectiveness of antagonist against pregnant mares' serum in three thyroidectomized rats.

In view of the importance of an understanding of the mechanism of the action of the 'antagonist' and of the question of its identity with the thyrotropic hormone, these implications in the concept of Tolksdorf and Jensen have been tested.

Received for publication October 4, 1940.

[1] Aided in part by a grant from the Rockefeller Foundation and administered by Dr. P. E. Smith, and in part by a grant from the Francis I. and Elizabeth C. Proctor Fund to one of us (G.K.S.).

[2] In most reports concerning the phenomenon of antagonism, the inhibiting material is injected intraperitoneally. However, Evans et al. (2) have obtained partial antagonism by subcutaneous injection. Bischoff (6), by decreasing the rate of absorption of the gonadotropic but not of the antagonistic principle, has also been able to demonstrate partial antagonistic activity of subcutaneously injected pituitary extract.

TABLE 1. RELATION OF THE METHOD OF ADMINISTRATION OF THYROTROPIC EXTRACTS TO THE THYROID WEIGHT RESPONSE IN GUINEA PIGS

	Number animals	Body weight $\pm\Sigma_m$	Thyroid weight $\pm\Sigma_m$
		gm.	*mg.*
No treatment	8	171.2±11.2 (125 to 215)	23.6±1.5 (17.7 to 28.6)
Intraperitoneal injections	11	159.1±21.9 (107 to 203)	47.2±9.7 (19.4 to 67.3)
Subcutaneous injections	9[1]	159.2±6.8 (130 to 190)	57.6±4.0 (41.0 to 72.8)

[1] The two heaviest animals died during treatment
Σ_m = mean deviation of the mean

EXPERIMENTAL

The following preparations were used. 1. An extract (*BAP21*) prepared as a flavianate from beef pituitary gland by methods previously described (12). This preparation, while crude, is well tolerated, even when given in relatively large amounts, and is rich in gonadotropic, adrenotropic, thyrotropic, and antagonistic activity.

2. A gonadotropic powder from the urine of postmenopausal women (*L630*) prepared by the method of Levin and Tyndale (13).

3. A gonadotropic preparation (*P9A*) from sheep pituitary glands partially fractionated by the method of Fevold (14). It contained follicle stimulating as well as luteinizing activity.

4. A commercial preparation of pregnant mares' serum gonadotropin (Antex Leo).

In order to test the effect of the route of administration on the response of the thyroid, 22 guinea pigs of 110 to 200 gm. body weight were injected daily for 5 days, each animal receiving a total of 6.1 mg. of the beef pituitary extract (*BAP21*). Eleven of the animals were injected subcutaneously once daily, and the other 11 received the same dose intraperitoneally. The animals were sacrificed 24 hours after the last injection and the thyroids dissected out and weighed. The thyroid weights (table 1) show that the extract was slightly less effective when given by intraperitoneal route.

The second group of experiments was designed to test the magnitude of the antagonism evoked in the absence of the thyroid gland. For this purpose 54 intact and 44 thyroidectomized immature female rats were used. Injections were begun when the animals were from 21 to 24 days of age, the operated animals having been thyroidectomized 2 to 4 days earlier. Injections were given twice daily for 3 days and the animals were sacrificed 96 hours after the first injection. At autopsy the ovaries were dissected out and weighed. Also, to verify completeness of thyroid ablation, the cervical region was examined under the dissecting microscope for traces of thyroid tissue. All suspected tissue was sectioned serially and examined microscopically. The few animals in which thyroid remnants were found were discarded from the series.

Both types (normal and thyroidectomized) of animals were divided into three groups. One group received the gonadotropin subcutaneously, another received the same subcutaneous gonadotropin treatment plus simultaneous intraperitoneal injections of the 'antagonist' (*BAP21*). The third group remained uninjected and served as controls. In the gonadotropin treatments the three types of extracts described above were used. Insofar as was possible, litter-mate animals were distributed among the 3 groups and between operated and unoperated animals.

The data obtained are given in table 2. It may be seen that the beef pituitary extract was a potent antagonist, particularly against the postmenopausal and pregnant

mares' serum gonadotropins, inhibiting the response of intact rats to these materials by 92 and 84% respectively. In thyroidectomized animals, treated identically, the antagonism was of the same order, being 96 and 75% respectively for the two gonadotropins.

The gonadotropic action of the sheep pituitary extract was not so effectively antagonized, the inhibition being 45 and 15% respectively in intact and thyroidectomized animals. The degree of antagonism was not increased by doubling the dosage of the antagonist.

DISCUSSION

As stated above, the proposal of Tolksdorf and Jensen (8) that the thyrotropic and antagonistic principles are identical requires the assumption that the thyrotropic hormone is more effective when administered by the intraperitoneal than by the subcutaneous route and that the phenomenon of antagonism cannot be evoked in the absence of the thyroid. The data of the present experiments show both of these assumptions to be incorrect.

The finding that thyrotropic hormone when administered subcutaneously is more effective than when given intraperitoneally (table 1) is not surprising. Presumably an extract placed in the peritoneal cavity passes into the circulation more rapidly than when injected subcutaneously and produces less prolonged and continuous stimulation. It has been shown (12) that thyrotropic extracts, like pituitary gonadotropic extracts (15), produce a greater response if the total dose is divided into multiple rather

TABLE 2. EFFECT OF THYROIDECTOMY ON 'ANTAGONISM'

Gonadotropin only				Gonadotropin plus antagonist				
Animals	Body weight		Ovarian weight	Animals	Body weight		Ovarian weight	Antagonism %[2]
	Start	End			Start	End		
	gm.	gm.	mg.		gm.	gm.	mg.	
Uninjected Controls								
16 normal	39	48	13.3±0.4					
9 thyect	37	45	13.0±1.0					
Post-Menopausal Urine Gonadotropin (L531)								
6 normal	37	43	48.9±11.9	6 normal	38	46	16.0±1.4	92.4
6 thyect	36	43	67.8±11.0	6 thyect	33	39	15.0±1.0	96.4
Increase in response due to thyroidectomy[1]			53.1%					
Pregnant Mare's Serum Gonadotropin (Antex)								
5 normal	36	45	164.6±25.5	4 normal	37	46	48.2±3.4	83.5
5 thyect	37	42	189.6±39.7	4 thyect	32	39	57.3±9.5	74.9
Increase in response due to thyroidectomy[1]			16.5%					
Sheep Pituitary Gonadotropin (P9A)								
9 normal	34	44	40.2±3.8	8 normal	35	43	28.1±3.1	45.0
9 thyect	37	43	68.1±6.3	6 thyect	36	44	59.7±4.1	15.2
Increase in response due to thyroidectomy[1]			103.7%					

[1] Increase in response due to thyroidectomy $= \frac{\text{ov. wt. (thyect)} - \text{ov. wt. (intact)}}{\text{ov. wt. (intact)} - \text{ov. wt. (control)}} \times 100.$

[2] % Antagonism $= \frac{\text{ov. wt. (no antag.)} - \text{ov. wt. (antag.)}}{\text{ov. wt. (no antag.)} - \text{ov. wt. (control)}} \times 100.$

than single daily injections. The fact that thyrotropic hormone, unlike the antagonist, is less effective when administered intraperitoneally does not support the concept of the identity of the two factors.

Although the inhibition of the action of certain types of gonadotropic material by the administration of thyroxin or thyroid gland substance has been demonstrated (9, 10) and the mechanism of this inhibition studied (11, 16), this mechanism does not appear to be involved in the pituitary-gonadotropic antagonism phenomenon as suggested by Tolksdorf and Jensen.

The data in table 2 show that gonadotropins from the pituitary gland, from pregnant mares' serum, and from the urine of postmenopausal women are inhibited by the 'antagonist' in the absence of the thyroid gland. Certainly no hyperthyroidism or increase in amount of thyroid secretion was induced in the thyroidectomized animals by the antagonistic extracts.

It is noteworthy that in these experiments all three gonadotropins gave a greater response (17 to 104%) in the thyroidectomized than in the normal test animals. This increased response in the absence of the thyroid, reported earlier by Schockaert (17) and by Leonard (18), when considered together with the equal effectiveness ofthe antagonist, indicates that the actual inhibition, as well as the gonadotropic action, is enhanced in the absence of the thyroid.

In experiments such as these, the distinctness of the pituitary factors is judged by the different responses they evoke. The data presented here do not in any way support the concept of the identity of the thyrotropic hormone and the factor which causes antagonism. They do show, however, that the phenomenon of antagonism is not mediated through the thyroid gland.

SUMMARY

The phenomenon of antagonism is evoked effectively in the absence of the thyroid gland as in intact animals when tested against gonadotropins from pituitary glands, postmenopausal urine, and from pregnant mares' serum. Therefore the phenomenon of antagonism is not mediated through the thyroid gland.

Thyrotropic extracts are slightly less effective when administered intraperitoneally than when given subcutaneously to guinea pigs.

The action of gonadotropic preparations of sheep hypophysis, postmenopausal urine and of pregnant mares' serum is considerably greater in thyroidectomized than in intact rats.

REFERENCES

1. Smith, P. E.: J.A.M.A. 88: 158. 1927.
2. Evans, H. M., K. Korpi, R. I. Pencharz and M. E. Simpson: *Univ. Calif. Pub. Anat.* 1: 237. 1936.
3. Leonard, S. L.: *Proc. Soc. Exper. Biol. & Med.* 31: 1157. 1934.
4. Fraenkel-Conrat, H., M. E. Simpson, C. H. Li and H. M. Evans: *Anales de la Facultad de Medicina, Montevideo* 25: 3. 1940.
5. Bunde, C. A., and A. A. Hellbaum: *Am. J. Physiol.* 125: 290. 1939.
6. Bischoff, F.: *J. Biol. Chem.* 133: 621. 1940.
7. Jensen, H., S. Tolksdorf and J. F. Grattan: *Am. J. Physiol.* 128: 532. 1940.
8. Tolksdorf, S., and H. Jensen: *Proc. Soc. Exper. Biol. & Med.* 42: 466. 1939.
9. Fluhmann, C. F.: *Am. J. Physiol.* 108: 498. 1934.
10. Tyndale, H. H., and L. Levin: *Am. J. Physiol.* 120: 486. 1937.
11. Smelser, G. K.: *Anat. Rec.* 73: 273. 1939.
12. Smelser, G. K.: *Endocrinology* 23: 429. 1938.
13. Levin, L., and H. H. Tyndale: *Proc. Soc. Exper. Biol. & Med.* 34: 516. 1936.
14. Fevold, H. L.: *Endocrinology* 24: 435. 1939.
15. Maxwell, L. C.: *Am. J. Physiol.* 110: 458. 1934.
16. Smelser, G. K.: *Anat. Rec. suppl.* 70: 63. 1937.
17. Schockaert, J.: *Compt. rend. Soc. de Biol.* 108: 431. 1931.
18. Leonard, S. L.: *Proc. Soc. Exper. Biol. & Med.* 34: 599. 1936.

EFFECT OF WATER ON THE M-DINITROBENZENE REACTION IN THE DETERMINATION OF CRYSTALLINE ANDROGENS BY THE EVELYN PHOTOELECTRIC COLORIMETER[1,2]

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THE TOTAL AMOUNT of a mixture of androgenic and non-androgenic ketonic substances is determined in the routine colorimetric assay of a urinary extract for its ketosteroid content. In these circumstances, which were discussed in a previous communication (1), the results of such an assay are best expressed in milligram equivalents of a fixed standard. The composition of such a standard must depend upon the chemical identity of the urinary androgens, on the relative proportions in which the individual androgens occur and on the reactions of each and of a mixture of all of them with m-dinitrobenzene in an alkaline alcoholic solution. The choice of the most suitable standard has been sub judice, since the only ketosteroids which have been isolated from and identified in normal male and female urine are androsterone, dehydroisoandrosterone and aetiocholane-3(α)-ol-17-one, and of these only the first two have been available for colorimetric study. Although these and any other ketosteroids which will be isolated from the urine possess the cyclopentanophenanthrene ring nucleus in common, slight differences in stereoisomeric and side-chain configuration and in degree or position of unsaturation may be enough to alter signifiantly their behavior with m-dinitrobenzene in an alkaline alcoholic solution. If the chromogenic properties of all ketosteroids, singly or in a mixture, were found to be identical under the experimental conditions of the m-dinitrobenzene reaction, the total ketosteroid content could be determined colorimetrically without knowing the relative proportions of the various substances in solution, and any one of the urinary androgens could be used as a standard substance of reference. If, however, there is a difference in the amount of color developed per unit of weight from the various ketosteroids, one might expect that the total ketosteroid content reported in milligram equivalents of any one of these substances would differ significantly from the actual amount present. Such a situation would necessitate the adoption of a standard which consists of a mixture of androgens in the proportions in which they occur in the urine. The results of urine assays would then be read from a calibration curve based on such a standard.

In an effort to secure evidence which would aid in the selection of a suitable standard, a series of observations have been made in the Evelyn photoelectric colorimeter of the chromogenic properties of crytalline androsterone, dehydroisoandrosterone and

Received for publication July 10, 1940.

[1] Aided by a grant from the Milton Fund, Harvard University.

[2] We are indebted to Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products, Inc., for the crystalline androsterone and dehydroisoandrosterone used in these experiments.

mixtures of these two in varying proportions. Such studies of crystalline androsterone and the method of its assay were reported in a previous paper (2). The present communication deals with a similar investigation of dehydroisoandrosterone and various mixtures of these two androgens. Aetiocholane-3(α)-ol-17-one will be studied similarly when it becomes available for experimentation.

Calibration with dehydroisoandrosterone. Four standard solutions of dehydroiso-

TABLE I

M	*G*	*L*	*C*	K_1	*M*	*G*	*L*	*C*	K_1
0.013	88^{2}	0.0518	0.0012	43.17	0.103	38^{3}	0.417	0.0097	42.99
0.013	87^{1}	0.0593	0.0012	49.42	0.103	38^{2}	0.412	0.0097	42.47
0.019	82^{1}	0.0848	0.0018	47.11	0.103	36^{3}	0.435	0.0097	44.85
7.019	82^{2}	0.0822	0.0018	45.67	0.103	37^{1}	0.429	0.0097	44.23
0.019	83^{0}	0.0809	0.0018	44.94	0.103	36^{2}	0.435	0.0097	44.85
0.019	81^{2}	0.0901	0.0018	50.06	0.103	37^{3}	0.423	0.0097	43.61
0.026	79^{2}	0.0982	0.0025	39.28	0.103	38^{0}	0.420	0.0097	43.30
0.026	75^{0}	0.1249	0.0025	49.96	0.115	34^{2}	0.462	0.0108	42.78
0.026	80^{1}	0.0955	0.0025	38.20	0.115	33^{2}	0.475	0.0108	43.98
0.026	75^{0}	0.1249	0.0025	49.96	0.115	33^{1}	0.478	0.0108	44.26
0.026	78^{2}	0.1037	0.0025	41.48	0.154	23^{0}	0.638	0.0145	44.00
0.026	74^{1}	0.1293	0.0025	51.72	0.154	23^{2}	0.629	0.0145	43.38
0.027	75^{2}	0.1206	0.0026	46.38	0.154	24^{2}	0.606	0.0145	41.79
0.027	77^{0}	0.1135	0.0026	43.65	0.154	24^{2}	0.606	0.0145	41.79
0.027	76^{2}	0.1163	0.0026	44.73	0.154	23^{2}	0.624	0.0145	43.03
0.027	75^{2}	0.1221	0.0026	46.96	0.154	24^{2}	0.611	0.0145	42.14
0.027	79^{0}	0.1024	0.0026	39.38	0.154	23^{2}	0.624	0.0145	43.03
0.027	78^{0}	0.1079	0.0026	41.50	0.154	23^{0}	0.638	0.0145	44.00
0.035	71^{3}	0.1442	0.0033	43.70	0.206	14^{1}	0.846	0.0194	43.61
0.035	72^{2}	0.1397	0.0033	42.33	0.206	14^{3}	0.831	0.0194	42.84
0.036	71^{0}	0.1487	0.0034	43.74	0.206	14^{2}	0.838	0.0194	43.20
0.036	71^{1}	0.1472	0.0034	43.29	0.246	9^{2}	1.022	0.0232	44.05
0.046	65^{1}	0.1855	0.0043	43.14	0.246	10^{0}	1.000	0.0232	43.10
0.046	64^{2}	0.1905	0.0043	44.30	0.246	10^{2}	0.979	0.0232	42.20
0.051	61^{1}	0.2129	0.0048	44.35	0.246	10^{1}	0.989	0.0232	42.63
0.051	61^{1}	0.2129	0.0048	44.35	0.246	10^{3}	0.969	0.0232	41.77
0.051	61^{3}	0.2093	0.0048	34.60	0.246	9^{2}	1.022	0.0232	44.05
0.051	61^{3}	0.2093	0.0048	43.60	0.247	10^{2}	0.979	0.0233	42.02
0.051	62^{1}	0.2059	0.0048	42.90	0.247	10^{1}	0.989	0.0233	42.45
0.051	62^{1}	0.2059	0.0048	42.90	0.247	9^{3}	1.011	0.0233	43.39
0.051	61^{1}	0.2129	0.0048	44.35	0.247	10^{0}	1.000	0.0233	42.92
0.077	48^{1}	0.317	0.0073	43.42	0.247	10^{1}	0.989	0.0233	42.45
0.077	48^{2}	0.314	0.0073	43.01	0.247	10^{2}	0.979	0.0233	42.02
0.077	48^{2}	0.314	0.0073	43.01	0.247	10^{1}	0.989	0.0233	42.45
0.077	47^{3}	0.321	0.0073	43.97	0.247	10^{1}	0.989	0.0233	42.45

M: Amount in mg. of androsterone in the total volume (10.6 cc.) of colored solution.

G: Corrected galvanometer reading (i.e., corrected for the slight individual variations of different galvanometers from true linearity).

L: Measured by the negative logarithm of the transmission of light (2—log *G*) and represents the density of the solution.

C: Amount of androsterone in mg. per cc. of colored solution and is numerically equal to *M*/10.6.

K_1: A constant which is numerically equal to *L*/*C*.

androsterone in 95% ethyl alcohol were prepared so as to contain 1.5, 0.75, 0.375, and 0.183 mg., respectively, of this substance per cc. of solution. Two-tenths of a cc. of each of these solutions, or a smaller volume diluted to 0.2 cc. with 95% ethyl alcohol, was treated in accordance with a previously described procedure of color development for use in the Evelyn photoelectric colorimeter (2). Calibration of this instrument with dehydroisoandrosterone consisted essentially in determining the values of K_1 by obtaining the galvanometer readings for known concentrations of the androgen. Such determinations were made in duplicate and checked several times with concen-

trations of dehydroisoandrosterone covering the readable range of the colorimeter. Three different but identically prepared batches of the solutions of dehydroisoandrosterone and 5 different but identically prepared batches of the reagents were used in these experiments.

The results in table 1 show that there is considerable variability in the data obtained with less than 0.035 mg. dehydroisoandrosterone. The values for K_1 were found to be constant, within limits of experimental error, in the range of 0.035 to 0.247 mg. dehydroisoandrosterone. Concentrations greater than the latter give read-

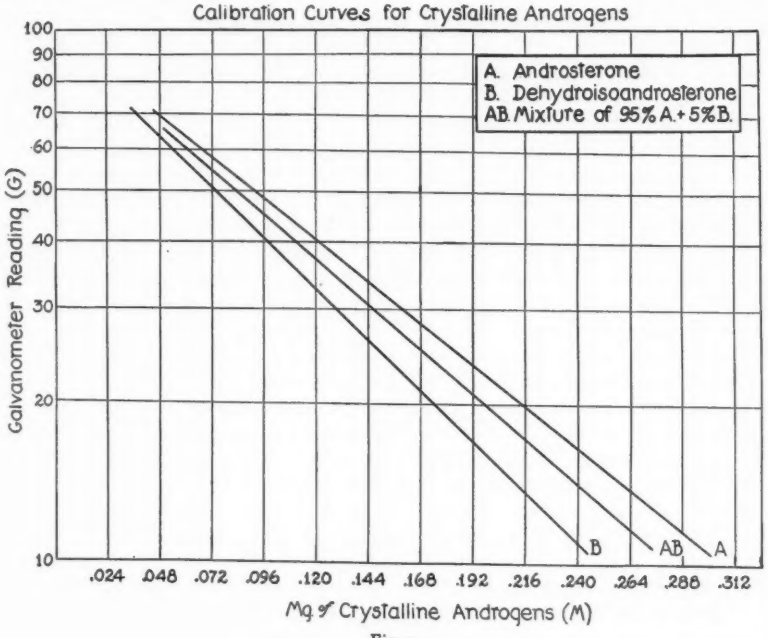

Fig. 1

ings outside the useable range of the instrument. The mean value of K_1 in the useful range of the galvanometer is 43.23 ± 0.80. This represents a coefficient of variation from the mean of $\pm 1.85\%$.

The calibration curve in figure 1A was constructed on the basis of the data in table 1. The best fitted line for these data[3] is represented by the regression equation $L = 4.03M + 0.003$ with a residual standard deviation of 0.012, where L, which represents the density of the solution, is measured by the negative logarithm of the transmission of light (2—log G) and M is the amount of dehydroisoandrosterone in milligrams in the total volume of solution (10.6 cc.)

Figure 1B represents a previously determined calibration curve for crystalline androsterone (2). A comparison of these two curves, and the average K_1 value for each, shows that dehydroisoandrosterone produces slightly more color per unit of weight than androsterone. On the basis of the two average K_1 values the ratio of color

[3] The best fitted line for the calibration data on crystalline androsterone is represented by the regression equation $L = 3.27$ M. A typographical error in this equation appeared in our previous paper in *Endocrinology* 25: 919. 1939.

developed per unit weight of dehydroisoandrosterone is to that of androsterone as 1.24 is to 1. These results confirm those previously obtained in a similar investigation by visual colorimetry in which the ratio was found to be 1.3 to 1(3) and are in accord also with the results of a spectrophotometric study of these two androgens (1).

That the color-developing power of the two androgens is not identical is not surprising in view of the fact that their chemical structures differ in the degree of unsaturation and in the stereoisomeric configuration of carbon atom 3. The method of color development used in these experiments is apparently sensitive enough to register this difference. Langstroth and Talbot (4), on the other hand, who used a modification of the Wu and Chou-Callow method (5), found that androsterone and dehydroisoandrosterone give precisely the same amount of color per unit weight of either hormone. The only significant difference between the method used in the present experiments and that of the Wu and Chou-Callow technic is the water content of the reaction mixture. Color development in the latter procedure takes place in absolute alcohol, whereas in the present method the reaction occurs in what is essentially a 65% alcoholic solution, viz., 0.2 cc. of a 2% solution of m-dinitrobenzene in absolute alcohol, 0.2 cc. of a 95% alcoholic solution of the hormones and 0.2 cc. of a 2N aqueous solution of KOH. These results and those of a previous extensive experience (1, 3, 6, 7) with the m-dinitrobenzene-aqueous KOH method differ completely from Neustadt's finding (8) that identical colors are developed per unit weight of substance when androsterone or dehydroisoandrosterone are assayed by a procedure in which water takes part in the chemical reaction.

The androsterone-dehydroisoandrosterone content of urine. These experimental observations have a distinct bearing on the application of the colorimetric method of assay to the determination of the total ketosteroid content of a urinary extract. The use of alcoholic KOH obviates the necessity of knowing the proportion of androsterone to dehydroisoandrosterone in a urine since they react similarly with m-dinitrobenzene under these experimental conditions. It is likewise clear, if aqueous KOH is used in the m-dinitrobenzene reaction, that it is desirable to have this information since dehydroisoandrosterone gives 1.24 times as much color per unit weight of substance as androsterone.

Studies of the α, β-ketosteroid content of normal female urine are already available. Callow (9) has found that the α-ketosteroid content (androsterone and aetiocholane-3(α)ol-17-one) is 2.6 mg. per liter as compared with 0.2 mg. per liter of the β-ketosteroid dehydroisoandrosterone. The latter, therefore, accounts for approximately 7% of the total of these androgens. Callow arrived at these results by quantitative isolation and chemical identification of the various androgens. These results are on the whole somewhat lower than those found by Talbot, Butler and MacLachlan (10) who report that the β-steroid fraction constitutes at least 10 to 15% of the total ketosteroid content of urine from normal adult males and females and children. These figures are based on digitonin precipitation of the dehydroisoandrosterone, the quantity of which was estimated by subtraction of the α-steroid content from the total ketosteroids of the urinary extract. The α and total ketosteroids were determined colorimetrically. In this procedure it is assumed that the β-steroids are unchanged by hydrolysis and are extracted quantitatively from the urine. However, in confirmation of Butenandt's findings (11) with strenuous acid hydrolysis, Wolfe (12) has recently found that even with 10-minute hydrolysis an appreciable amount of dehydroisoandrosterone is converted to its chloro- derivative (chlorodehydroisoandrosterone). It is possible that simultaneous hydrolysis and extraction may protect the dehydroisoandrosterone against this effect which may have repercussions in the colorimetric evaluation of the ketosteroid content of urine extracts. This factor must be taken into

consideration in the interpretation of Talbot's results since the chloro- derivative is not precipitated by digitonin but comes over in the α-steroid fraction. Even with these corrections, however, it is probable that the total β-steroid content of normal urine is less than 30% of the total urinary ketosteroids which, as other experiments indicate (table 2) is an important factor in this connection. A similar study has not yet been made of the β-steroid content of the urine of hirsute patients, but Callow (13) and

TABLE 2

Composition of mixture					G	L			"Total androgen" in mg. equivalents of androsterone	% deviation from known "total androgen"	C	K_1
Androsterone		Dehydroisoandrosterone		Milligrams of "total androgen"		Observed	Theoretical	% difference				
mg.	% of total	mg.	% of total									
0.046	90.2	0.005	9.8	0.051	64^2	0.191			0.057	11.8	0.0048	39.68
0.046	90.2	0.005	9.8	0.051	63^2	0.196			0.058	13.7	0.0048	40.75
0.046	90.2	0.005	9.8	0.051	64^1	0.192			0.058	11.8	0.0048	40.04
0.046	90.2	0.005	9.8	0.051	63^2	0.197			0.059	15.7	0.0048	41.10
0.042	79.3	0.011	20.8	0.053	62^2	0.202			0.061	15.1	0.0050	40.48
0.042	79.3	0.011	20.8	0.053	62^2	0.202			0.061	15.1	0.0050	40.48
0.042	79.3	0.011	20.8	0.053	62^0	0.208			0.063	18.9	0.0050	41.52
0.042	79.3	0.011	20.8	0.053	63^0	0.201			0.060	13.1	0.0050	40.14
0.050	94.3	0.003	5.7	0.053	62^2	0.204			0.061	15.1	0.0050	40.80
0.050	94.3	0.003	5.7	0.053	63^2	0.196			0.058	9.4	0.0050	39.12
0.050	94.3	0.003	5.7	0.053	63^2	0.196			0.058	9.4	0.0050	39.12
0.050	94.3	0.003	5.7	0.053	64^1	0.192			0.058	8.5	0.0050	38.44
0.100	95.2	0.005	4.8	0.105	43^2	0.362			0.109	3.8	0.0099	36.77
0.100	95.2	0.005	4.8	0.105	43^1	0.364			0.110	4.8	0.0099	36.57
0.100	95.2	0.005	4.8	0.105	43^2	0.369			0.112	6.7	0.0099	36.77
0.100	95.2	0.005	4.8	0.105	43^1	0.364			0.110	4.8	0.0099	37.27
0.100	90.9	0.010	9.1	0.110	39^2	0.401			0.121	10.0	0.0104	38.56
0.100	90.9	0.010	9.1	0.110	41^1	0.385					0.0104	37.02
0.100	90.9	0.010	9.1	0.110	41^0	0.387			0.117	6.4	0.0104	37.21
0.100	90.9	0.010	9.1	0.110	40^2	0.390			0.118	7.3	0.0104	37.50
0.100	84.0	0.019	16.0	0.119	38^1	0.417			0.127	6.7	0.0112	37.23
0.100	84.0	0.019	16.0	0.119	37^2	0.426			0.130	8.4	0.0112	38.03
0.100	84.0	0.019	16.0	0.119	37^1	0.429			0.130	9.2	0.0112	38.30
0.100	84.0	0.019	16.0	0.119	37^2	0.423			0.128	7.6	0.0112	37.77
0.100	76.3	0.031	23.7	0.131	34^2	0.459			0.139	6.1	0.0123	37.32
0.100	76.3	0.031	23.7	0.131	34^0	0.469			0.142	8.4	0.0123	38.13
0.100	76.3	0.031	23.7	0.131	34^2	0.459			0.139	6.1	0.0123	37.32
0.100	76.3	0.031	23.7	0.131	33^2	0.472			0.143	9.2	0.0123	38.37
0.100	71.4	0.040	28.6	0.140	32^2	0.485	0.496	2.2	0.147	5.0	0.0132	36.74
0.100	71.4	0.040	28.6	0.140	32^2	0.488	0.496	1.6	0.149	6.4	0.0132	36.97
0.100	71.4	0.040	28.6	0.140	31^2	0.498	0.496	0.4	0.151	7.9	0.0132	37.73
0.100	71.4	0.040	28.6	0.140	31^2	0.498	0.496	0.4	0.151	7.9	0.0132	37.73
0.146	90.7	0.015	9.3	0.161	26^1	0.581			0.177	9.9	0.0152	38.22
0.146	90.7	0.015	9.3	0.161	27^1	0.565			0.172	6.8	0.0152	37.17
0.146	90.7	0.015	9.3	0.161	26^0	0.585			0.178	10.6	0.0152	38.49
0.146	90.7	0.015	9.3	0.161	27^0	0.569			0.174	8.1	0.0152	37.43
0.131	80.9	0.031	19.1	0.162	26^1	0.581			0.177	9.3	0.0152	38.22
0.131	80.9	0.031	19.1	0.162	26^1	0.581			0.177	9.3	0.0152	38.22
0.131	80.9	0.031	19.1	0.162	26^1	0.581			0.177	9.3	0.0152	38.22
0.131	80.9	0.031	19.1	0.162	27^1	0.565			0.172	6.2	0.0152	37.17
0.154	95.1	0.008	4.9	0.162	24^1	0.598			0.182	12.3	0.0153	39.08
0.154	95.1	0.008	4.9	0.162	24^1	0.598			0.182	12.3	0.0153	39.08
0.154	95.1	0.008	4.9	0.162	26^1	0.581			0.177	9.3	0.0153	37.97
0.154	95.1	0.008	4.9	0.162	25^2	0.589			0.179	10.5	0.0153	38.50
0.216	80.0	0.054	20.0	0.270	11^2	0.930	0.926	0.4	0.284	5.2	0.0255	36.47
0.216	80.0	0.054	20.0	0.270	11^1	0.949	0.926	2.5	0.290	7.4	0.0255	37.22
0.216	80.0	0.054	20.0	0.270	11^1	0.949	0.926	2.5	0.290	7.4	0.0255	37.22
0.216	80.0	0.054	20.0	0.270	11^2	0.939	0.926	1.4	0.287	6.3	0.0255	36.82
0.262	96.0	0.011	4.0	0.273	11^2	0.930			0.285	4.4	0.0258	36.17
0.262	96.0	0.011	4.0	0.273	11^0	0.959			0.293	7.3	0.0258	37.17
0.262	96.0	0.011	4.0	0.273	11^1	0.949			0.291	6.6	0.0258	36.78
0.262	96.0	0.011	4.0	0.273	11^2	0.930			0.285	4.4	0.0258	36.17
0.246	88.8	0.031	11.2	0.277	10^2	0.979			0.299	7.9	0.0261	37.51
0.246	88.8	0.031	11.2	0.277	10^2	0.969			0.296	6.9	0.0261	37.12
0.246	88.8	0.031	11.2	0.277	9^2						0.0261	39.16
0.246	88.8	0.031	11.2	0.277	10^0	1.000			0.306	10.5	0.0261	38.31

Friedgood and Wolfe (14) have studied the urine of patients with virilism due to corticoadrenal tumors from this viewpoint. Callow traced more than 70% of the androgenic activity of the urine of a patient with corticoadrenal tumor to dehydroisoandrosterone; and Wolfe has found that more than 50% of the ketosteroid content of the urine of a patient with a similar condition is in the form of dehydroisoandrosterone.

TABLE 3

Composition of mixture					G	L			Total androgen in mg. equivalents of androsterone	% deviation from known total androgen	C	K_1
Androsterone		Dehydroisoandrosterone		Total androgen, mg.		Observed	Theoretical	% difference				
mg.	% of total	mg.	% of total									
0.019	50.0	0.019	50.0	0.038	70^2	0.1518			0.046	21.1	0.0036	42.17
0.019	50.0	0.019	50.0	0.038	70^2	0.1518			0.046	21.1	0.0036	42.17
0.019	50.0	0.019	50.0	0.038	71^2	0.1457			0.045	18.4	0.0036	40.47
0.019	50.0	0.019	50.0	0.038	70^0	0.1549			0.047	23.7	0.0036	43.03
0.026	50.0	0.025	50.0	0.051	62^1	0.2059			0.062	21.6	0.0048	42.89
0.026	50.0	0.025	50.0	0.051	61^2	0.2111			0.064	25.5	0.0048	43.98
0.026	50.0	0.025	50.0	0.051	61^2	0.2111			0.064	25.5	0.0048	43.98
0.026	50.0	0.025	50.0	0.051	62^2	0.2024			0.060	17.6	0.0048	42.17
0.031	59.6	0.021	40.4	0.052	62^0	0.2076			0.063	21.2	0.0049	42.36
0.031	59.6	0.021	40.4	0.052	62^2	0.2041			0.061	17.3	0.0049	41.65
0.031	59.6	0.021	40.4	0.052	61^1	0.2129			0.064	23.1	0.0049	43.45
0.031	59.6	0.021	40.4	0.052	61^2	0.2093			0.063	21.2	0.0049	42.71
0.039	69.6	0.017	30.4	0.056	60^1	0.2200			0.066	17.9	0.0053	41.51
0.039	69.6	0.017	30.4	0.056	60^2	0.2164			0.065	16.1	0.0053	40.83
0.039	69.6	0.017	30.4	0.056	60^1	0.2200			0.066	17.9	0.0053	41.51
0.039	69.6	0.017	30.4	0.056	60^2	0.2182			0.065	16.1	0.0053	41.17
0.039	50.0	0.038	50.0	0.077	51^2	0.2882			0.087	13.0	0.0073	39.48
0.039	50.0	0.038	50.0	0.077	50^2	0.295			0.088	14.3	0.0073	40.41
0.039	50.0	0.038	50.0	0.077	51^2	0.2882			0.087	13.0	0.0073	39.48
0.039	50.0	0.038	50.0	0.077	51^2	0.2861			0.086	11.7	0.0073	39.19
0.039	50.0	0.038	50.0	0.077	51^2	0.2882			0.087	13.0	0.0073	39.48
0.039	50.0	0.038	50.0	0.077	51^2	0.2882			0.087	13.0	0.0073	39.48
0.051	48.6	0.054	51.4	0.105	40^1	0.395	0.392	0.8	0.120	14.3	0.0099	39.90
0.051	48.6	0.054	51.4	0.105	40^1	0.395	0.392	0.8	0.120	14.3	0.0099	39.90
0.100	64.9	0.054	35.1	0.154	26^0	0.585	0.552	6.0	0.178	15.6	0.0145	40.34
0.100	64.9	0.054	35.1	0.154	25^2	0.594	0.552	7.7	0.181	17.5	0.0145	40.96
0.100	64.9	0.054	35.1	0.154	25^2	0.589	0.552	6.7	0.179	16.2	0.0145	40.62
0.100	64.9	0.054	35.1	0.154	26^0	0.585	0.552	6.0	0.178	15.6	0.0145	40.34
0.077	50.0	0.077	50.0	0.154	24^3	0.606	0.570	6.3	0.185	20.1	0.0145	41.79
0.077	50.0	0.077	50.0	0.154	24^2	0.611	0.570	7.2	0.186	20.8	0.0145	42.14
0.077	50.0	0.077	50.0	0.154	25^1	0.598	0.570	4.9	0.182	18.2	0.0145	41.24
0.077	50.0	0.077	50.0	0.154	25^2	0.594	0.570	4.2	0.181	17.5	0.0145	40.97
0.077	50.0	0.077	50.0	0.154	25^1	0.598	0.570	4.9	0 182	18.2	0.0145	41.24
0.077	50.0	0.077	50.0	0.154	26^1	0.581	0.570	1.9	0.177	14.9	0.0145	39.31
0.100	61.7	0.062	38.3	0.162	23^3	0.624	0.584	6.8	0.190	17.3	0.0153	40.78
0.100	61.7	0.062	38.3	0.162	23^2	0.629	0.584	7.5	0.192	18.5	0.0153	41.11
0.100	61.7	0.062	38.3	0.162	24^3	0.606	0.584	3.8	0.185	14.2	0.0153	39.61
0.100	61.7	0.062	38.3	0.162	23^3	0.624	0.584	6.8	0.190	17.3	0.0153	40.78
0.116	69.5	0.051	30.5	0.167	23^3	0.624	0.593	5.2	0.190	13.8	0.0158	39.49
0.116	69.5	0.051	30.5	0.167	23^2	0.629	0.593	6.1	0.192	15.0	0.0158	39.81
0.116	69.5	0.051	30.5	0.167	24^2	0.611	0.593	3.0	0.186	11.4	0.0158	38.67
0.116	69.5	0.051	30.5	0.167	23^1	0.634	0.593	6.9	0.193	15.6	0.0158	40.13
0.085	49.7	0.086	50.3	0.171	22^1	0.653	0.631	3.5	0.199	16.4	0.0161	40.56
0.085	49.7	0.086	50.3	0.171	22^1	0.653	0.631	3.5	0.199	16.4	0.0161	40.56
0.085	49.7	0.086	50.3	0.171	22^0	0.658	0.631	4.3	0.201	17.5	0.0161	41.49
0.085	49.7	0.086	50.3	0.171	23^2	0.643	0.631	1.9	0.196	14.6	0.0161	39.94
0.092	50.0	0.092	50.0	0.184	19^3	0.704	0.679	3.7	0.215	16.8	0.0174	40.46
0.092	50.0	0.092	50.0	0.184	19^2	0.704	0.679	3.7	0.215	16.8	0.0174	40.46
0.092	50.0	0.092	50.0	0.184	19^2	0.710	0.679	4.6	0.217	17.9		
0.185	69.3	0.082	30.7	0.267	11^1	0.949	0.938	1.2	0.290	8.6	0.0252	37.66
0.185	69.3	0.082	30.7	0.267	11^0	0.959	0.938	2.2	0.293	9.7	0.0252	38.06
0.185	69.3	0.082	30.7	0.267	10^2	0.979	0.938	4.4	0.301	12.7	0.0252	38.85
0.185	69.3	0.082	30.7	0.267	10^2	0.969	0.938	3.3	0.295	10.5	0.0252	38.46
0.139	50.0	0.139	50.0	0.278	8^2							
0.139	50.0	0.139	50.0	0.278	9^1							
0.169	60.4	0.111	39.6	0.280	9^0							
0.169	60.4	0.111	39.6	0.280	9^2							
0.169	60.4	0.111	39.6	0.280	9^2							
0.169	60.4	0.111	39.6	0.280	8^2							

With these facts in mind, a study was made of the colorimetric behavior of various mixtures of crystalline androsterone and dehydroisoandrosterone in the m-dinitrobenzene-aqueous KOH reaction.

Study of the colorimetric behavior of various mixtures of crystalline androsterone and dehydroisoandrosterone in the m-dinitrobenzene reaction. Mixtures of the two crystalline androgens were prepared so that readings of the results fell in the lower, middle, and upper portions of the useable range of the galvanometer scale. The proportions of androsterone to dehydroisoandrosterone which were prepared varied from 95-5 to 50-50. Observations with these mixtures were made with two objectives in mind, viz., to ascertain whether or not the presence in solution of one of these androgens affects the rate and intensity of color development of the other, and to determine whether

or not the observed "total androgen" of such mixtures (reported in milligram equivalents of crystalline androsterone) compares favorably with its known androgen content.

The effect of one androgen of a mixture upon the color developing properties of the other. The observed L value (optical density) for any given mixture of androsterone and dehydroisoandrosterone should be equal to the sum of the theoretical L values for each of the androgens in solution if one of them does not affect the chromogenic properties of the other. Theoretical L values were calculated for those mixtures in which the amounts of each of the component androgens fell within the range covered by their respective calibration curves. In the 39 samples thus studied, the percentage differences between the observed and theoretical L values ranged from less than 1 to 7.6 with an average of 3.9% (table 2, 3). This difference is only slightly greater than

TABLE 4

Percentage composition of androgen mixture		Average K_1 value
Androsterone	Dehydroisoandrosterone	
100	0	34.72±0.68
95–85	5–15	38.22±1.3
80–70	20–30	38.71
69–60	31–40	41.23
50	50	41.01
0	100	43.23±0.80

the average experimental error of approximately 2% which is encountered in the determination of either androsterone or dehydroisoandrosterone alone. One may conclude, therefore, that the chromogenic property of one of these androgens is altered only to a negligible extent, if at all, by the addition of the other to the solution.

Comparison of observed "total androgen" (in mg. equivalents of androsterone) with the known ketosteroid content of mixtures of androsterone and dehydroisoandrosterone. The "total androgen" value of each of the various mixtures was determined from its color content and recorded in milligram equivalents of crystalline androsterone. These equivalents were read off from the calibration curve for androsterone in the manner adopted for the assay of the chromogen content of a urinary extract. The results in table 2 indicate that one may expect an average difference of 7.7% between the observed and known amounts of "total androgen" in a mixture of crystalline androsterone and dehydroisoandrosterone if two conditions are fulfilled. When either one or both of these conditions are violated, the average difference increases to 16.6% (table 3). The two conditions are as follows.

(*a*) That each of the androgens is present in solution in amounts which can be determined from its calibration curve in accordance with Beer's law.

(*b*) That any given mixture of these androgens contains not more than 30% dehydroisoandrosterone because the amount of color developed in the present modification of the m-dinitrobenzene reaction is somewhat more per unit weight of dehydroisoandrosterone than per unit weight of androsterone.

The behavior of mixtures of these 2 crystalline androgens in the m-dinitrobenzene-aqueous KOH reaction is precisely what one might expect from similar studies of the individual hormones. These results obtained with the m-dinitrobenzene-aqueous KOH method are in distinct contrast to those reported by Langstroth and Talbot (4) who worked with a modification of the Wu and Chou-Callow alcoholic KOH method and found that mixtures of androsterone and dehyroisoandrosterone behave colorimetrically like simple solutions of either androgen.

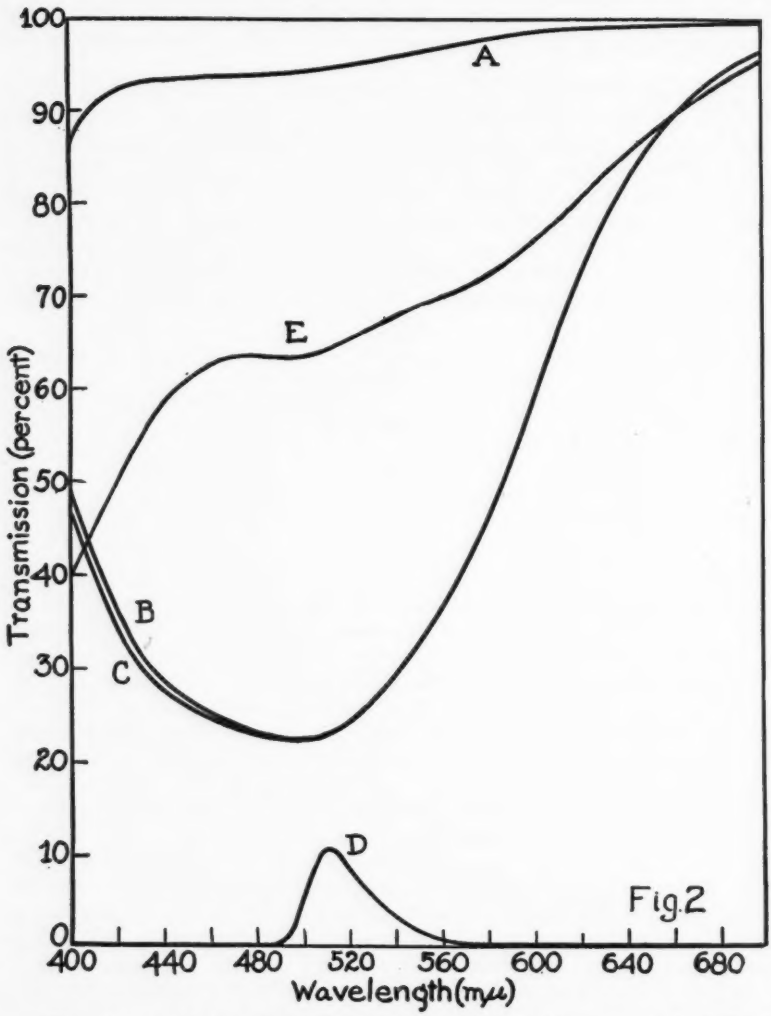

Fig. 2. *A*, spectrophotometric curve for the blank solution containing reagents only. *B*, *C* and *E*, spectrophotometric curves for the colors developed in solutions with androsterone, dehydroisoandrosterone and testosterone, respectively, by the aqueous KOH modification of the m-dinitrobenzene reaction described in this paper. *D*, spectrophotometric curve of No. 520 filter of the Evelyn photoelectric colorimeter.

■ *Calibration of the photoelectric colorimeter with various mixtures of androsterone and dehydroisoandrosterone.* The K_1 values, which were determined for 95·5 to 70·30 mixtures of androsterone to dehydroisoandrosterone, are essentially identical (table 4) and fall between the K_1 values for each of the pure crystalline androgens. K_1 values for mixtures containing more than 30% dehydroisoandrosterone are somewhat higher and therefore closer to the K_1 value of crystalline dehydroisoandrosterone than to that of androsterone (table 4). Accordingly, the calibration curves for these mixtures fall

between those constructed for the individual crystalline androgens (fig. 1 AB). As one might expect, these results differ from those of Langstroth and Talbot (4) who find identical K_1 values for both androgens with the Wu and Chou-Callow absolute alcohol technic.

Application of calibration values to the assay of urinary ketosteroids. On the basis of this evidence it is clear that the m-dinitrobenzene-alcoholic KOH method is preferable to that of the m-dinitrobenezene-aqueous KOH for the quantitative assay of simple mixtures of crystalline androsterone and dehydroisoandrosterone. However, the determination of the ketosteroid content of urine extracts presents a somewhat more complex problem. There is no assurance, for example, that the colorimetric behavior of all of the urinary ketosteroids is identical in the m-dinitrobenzene reaction, nor that one or more of them would not affect the rate and intensity of color development of

TABLE 5

Source of specimen	Crystalline androgen or mixture of androgens for which calibration curve was determined			
	100% A[1]	95% A+5% DA[2]	100% DA[3]	% difference
Urine of normal adult women	7.5	6.7		9.8
	9.9	9.0		9.6
	11.9	10.9		8.8
	14.2	12.9		9.2
Urine of women with hirsutism but no virilism	13.5	12.3		9.2
	16.1	14.9		7.6
	17.8	16.2		9.0
	19.9	18.2		8.9
Urine of a 10-year-old girl with virilism due to corticoadrenal tumor	218.8		186.5	18.0
	269.8		219.9	18.1
	388.6		315.7	18.8

[1] Results reported in terms of mg. equivalents crystalline androsterone per 24 hours.
[2] Results reported in terms of mg. equivalents per 24 hours of this mixture of crystalline androsterone and dehydroisoandrosterone.
[3] Results reported in terms of mg. equivalents of crystalline dehydroisoandrosterone per 24 hours.

the others present in the urine extract. Judging from the behavior of testosterone, all ketosteroids do not react similarly with m-dinitrobenzene even in the Wu and Chou-Callow alcoholic KOH method. Figure 2 shows the striking difference between the spectrophotometric curves of the colors produced in the m-dinitrobenzene reaction by androsterone and testosterone, both of which are ketosteroids. Langstroth and Talbot (15), moreover, have shown that testosterone inhibits the color producing properties of androsterone when simple mixtures of the two are treated with m-dinitrobenzene in an alcoholic solution of KOH. The fact that testosterone, although not a constituent of urine, has different color producing properties than androsterone or dehydroisoandrosterone suggests that complex mixtures of urinary ketosteroids, some of which are as yet unidentified, cannot be regarded in the same light as simple mixtures of these 2 crystalline androgens until data in support of this hypothesis are available.

Additional evidence bearing on the same point indicates that the position of the keto group affects the rate and intensity of the color development. Callow, Callow and Emmens (5) have found that neutral fractions of extracts of hydrolyzed urine give the color reactions characteristic of 17-ketosteroids, although there is some indication, spectrophotometrically, of the presence of other chromogenic compounds. Qualitative evidence, from the rate of color development with various urine extracts, suggests that substances like saturated 3-ketosteroids are present in small amounts.

The foregoing remarks indicate to some extent the complexity of the problem involved in the colorimetric determination of the ketosteroid content of a urine extract. From a theoretical viewpoint one could calculate the extent to which the known difference in color producing properties of androsterone and dehydroisoandrosterone may affect the accuracy of the determinations made with the aqueous-alcoholic method (table 5). The calibration curves from which the calculations in table 5 were made are based on the relative proportions of dehydroisoandrosterone found in the urines of normal women and of patients with virilism due to corticoadrenal tumor. Since the K_1 values for mixtures of androsterone and dehydroisoandrosterone containing 5 to 30% of the latter are essentially the same, one might report the urinary ketosteroids of normal individuals in terms of a calibration curve based on the 95-5 mixture of androsterone and dehydroisoandrosterone. The average K_1 value for this mixture is 38.22 ± 1.3 with a coefficient of variation from the mean of 3.5%. The best fitted line for these data is represented by the regression equation $L = 3.49M + 0.01$. Similar reasoning suggests that a calibration curve based on either dehydroisoandrosterone or a 50-50 mixture of the latter with androsterone might be used in the assay of the urinary ketosteroids of patients with corticoadrenal tumors. Table 5 shows that the more nearly correct value for the ketosteroid content of the urine of a patient with corticoadrenal tumor is about 18% less, and that of a normal female is approximately 9% less than the results which are calculated in terms of crystalline androsterone alone. From a practical standpoint, however, it would be more accurate to determine the correction factor for the aqueous alcoholic method by more direct means. This has been done in conjunction with other observations and is being reported in a forthcoming communication (16).

The results obtained either with the aqueous KOH or the absolute alcoholic KOH methods lead to similar correlations between urinary ketosteroids and the clinical condition of the individuals who are being studied because the range of the ketosteroid excretion in any one group of patients is rather large (17). There is a minor advantage in using the aqueous KOH method for routine clinical determinations in that this obviates the necessity of dissolving KOH in alcohol at freezing temperatures in a relatively low humidity and does away with the frequent need of making up fresh solutions of alcoholic KOH which cannot be used after several days because of the undesirable color development in the blank. So far as is known at present, however, the alcoholic KOH method gives a more accurate indication of the ketosteroid content of a mixture of the substances, and is for this reason to be preferred to the aqueous KOH method.

It is obviously highly desirable to standardize the colorimetric method of ketosteroid assay so that all laboratories working with it use essentially the same procedure with unimportant modifications adapted to the instrument with which the readings are made. For reasons which have been recorded in this paper, and because of other pertinent observations which are being included in another communication (16) it is suggested that the Wu and Chou-Callow alcoholic KOH method is adopted universally for such determinations. If further investigation shows that the colorimetric behavior of certain urinary androgens, as yet untested, differ significantly enough from that of androsterone and dehydroisoandrosterone, it may be desirable to modify the composition of the fixed standard in terms of which the results are reported. Until then, crystalline androsterone alone appears to be an acceptable standard substance of reference.

SUMMARY

The presence or absence of water influences the colorimetric reaction of androsterone and dehydroisoandrosterone or various mixtures of the two with m-dinitroben-

zene in an alkaline alcoholic solution. Dehydroisoandrosterone yields more color per unit of weight than androsterone (1.24 to 1) when the reaction is carried out in 2N aqueous KOH. When the color reaction is carried out in absolute alcohol, however, these androgens give the same amount of color per unit weight of substance. In the latter circumstance, mixtures of the two androgens may be treated as simple solutions of one or the other substance from a chromogenic viewpoint. This rule does not apply to solutions in which aqueous KOH is used in the reaction mixture. In the latter case, if the "total androgen" of a mixture of the two is reported in mg. equivalents of androsterone, there will be an error which will depend in part on the difference in the chromogenic properties of the individual androgens. Hence, the error would be proportional to the amount of dehydroisoandrosterone in relation to the androsterone present in the mixture. Another factor which may play an important role in magnifying this error in the determination of the "total androgen" in a mixture of these substances is the total amount of each androgen in solution. In the interests of greater accuracy each of the androgens should be present in solution in amounts which can be determined from their respective calibration curves in accordance with Beer's law.

Of what practical significance are these differences in technic? The results obtained by the use of aqueous KOH in the m-dinitrobenzene reaction are higher in individual instances than those reported for corresponding groups of patients by Callow, Callow and Emmens who used only absolute alcohol in the reaction mixture; both series of results, however, lie within the same range. Consequently the differences in the results obtained by these two technics are not significant from a clinical viewpoint. From a chemical standpoint, however, the use of alcoholic KOH represents a refinement in technic and leads to more nearly quantitative results. The universal adoption of the Wu and Chou-Callow method in one of its acceptable modifications is urged in order to standardize the colorimetric procedure for the determination of the ketosteroid content of urine extracts.

REFERENCES

1. Friedgood, H. B., and H. L. Whidden: *Endocrinology* 27: 258. 1940.
2. Friedgood, H. B., and H. L. Whidden: *Endocrinology* 25: 919. 1939.
3. Friedgood, H. B., and H. L. Whidden: *Endocrinology* 27: 249. 1940.
4. Langstroth, G. O., and N. B. Talbot: *J. Biol. Chem.* 129: 759. 1939.
5. Callow, N. H., R. K. Callow and C. W. Emmens: *Biochem. J.* 32: 1312. 1938.
6. Friedgood, H. B., and H. L. Whidden: *New Eng. J. Med.* 220: 736. 1939.
7. Friedgood, H. B., and H. L. Whidden: *Endocrinology* 27: 242. 1940.
8. Neustadt, R.: *Endocrinology* 23: 711. 1938.
9. Callow, N. H., and R. K. Callow: *Biochem. J.* 33: 931. 1939.
10. Talbot, N. B., A. N. Butler and E. MacLachlan: *New Eng. J. Med.* (in press).
11. Butenandt, A., et al.: *Ztschr. f. physiol. Chem.* 229: 192. 1934; 237: 57. 1935.
12. Wolfe, J. K.: personal communication.
13. Callow R. K.: *J. Soc. Chem. Ind.* 55: 1030. 1936.
14. Friedgood, H. B., and J. K. Wolfe: *J. Clin. Investig.* 19: 779. 1940.
15. Langstroth, G. O., and N. B. Talbot: *J. Biol. Chem.* 128: 759. 1939.
16. Friedgood, H. B., and R. A. Berman: *Endocrinology* 28: 248. 1941.
17. Friedgood, H. B., and H. L. Whidden: Urinary Ketosteroids in Various Endocrinopathies. In preparation.

EFFECT OF WATER ON ACCURACY OF M-DINITROBENZENE REACTION IN THE QUANTITATIVE ASSAY OF URINARY KETOSTEROIDS[1]

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ZIMMERMANN's m-dinitrobenzene reaction has been generally accepted as the simplest and most expedient chemical procedure available at present for the quantitative assay of ketosteroids (1, 2). The obscurity of the chemistry involved in this colorimetric reaction, the instability of the reaction due to the numerous physical factors which affect it, and the use of several different types of colorimeters have led to the development by various laboratories of a number of arbitrary adaptations of this procedure (3–10). It was demonstrated in a previous investigation (11) that the essential difference between these various modifications of the Zimmermann reaction is in the water content of the reaction mixture. As a result of experience with the original Zimmermann procedure and all of its currently available adaptations (9–13), we suggested the desirability (13) of replacing it by some other chemical method which obviates its undesirable features. For practical clinical purposes, however, Zimmermann's m-dinitrobenzene reaction is adequate for the determination of the ketosteroid content of urine extracts. The present unsatisfactory state of the literature concerned with this field in which constantly appearing reports cannot be adequately compared with each other because of significant differences in technics suggests the necessity for standardizing this procedure. With this in mind, a comparative study of the various modifications of the m-dinitrobenzene reaction has been made in order to ascertain which of them gives the most quantitatively accurate account of the ketosteroid content of urine extracts and of mixtures of crystalline androsterone and dehydroisoandrosterone. In a previous investigation (11) it was demonstrated that the use of the m-dinotrobenzene-aqueous KOH method entails an average error of 2% in the quantitative determination of crystalline androsterone or dehydroisoandrosterone, an average error of 7.7% in the assay of mixture of these ketosteroids in which the dehydroisoandrosterone content is less than 30%, and an average error of 16.6% in the assay of such mixtures containing more than 30% of dehydroisoandrosterone. It was found also that an average error of 16.6% is introduced into these determinations if one assays a mixture of these 2 ketosteroids in which one or both of them are present in amounts which are not represented on their respective calibration curves.

The present series of observations was undertaken after it was discovered that doubling the amount of urine extract used for assay by this method did not give double the intensity of color which might have been expected on the basis of Beer's

Received for publication July 10, 1940.

[1] Aided by grants from the Milton Fund of Harvard University, from the Penrose Fund of the American Philosophical Society and from the Armour Laboratories.

law from a 2-fold increase in the amount of ketosteroids reacting with m-dinitrobenzene in the aqueous KOH medium.

Chemical procedures. Extracts of urine, collected from women and men with various endocrine disorders, were prepared by the procedure described in earlier work (9). The neutral ketosteroid-containing fractions were made up to a volume of 5 cc., 10 cc., or 25 cc. with 95% ethyl alcohol. Colorimetric determinations were made by the two modifications of the m-dinitrobenzene reaction which have been adapted for use in the Evelyn photoelectric colorimeter, viz., Friedgood and Whidden's method which is a modification of the original Zimmermann aqueous KOH procedure and Langstroth and Talbot's adaptation to this colorimeter of the Wu and Chou-Callow absolute alcohol modification of Zimmermann's reaction. The same urines were extracted for both series of colorimetric determinations.

Aqueous alcohol method. Various aliquot portions of the alcoholic extracts described above were pipetted into Evelyn colorimeter tubes for the determination of their ketosteroid content, but the volume of these solutions actually taking part in the chemical reaction was always adjusted to 0.2 cc. If less than 0.2 cc. of urine extract were used, the volume was diluted to 0.2 cc. with 95% alcohol; if more than 0.2 cc. were used, the extract was evaporated to dryness over a steam bath under reduced pressure and 0.2 cc. 95% alcohol was added to the residue. To each of such alcoholic extracts were then added 0.2 cc. 2% m-dinitrobenzene in absolute alcohol and 0.2 cc. 2N KOH. The reaction mixture was placed in semi-darkness in a water bath at 25°C. and kept there fore 75 minutes with occasional shaking. At the end of this time 10 cc. of 95% alcohol were added to the reaction mixture and the color was read immediately in the Evelyn photoelectric colorimeter with a No. 520 filter. The results of these observations were reported in terms of milligram equivalents crystalline androsterone per 24 hours.

Absolute alcohol method. A portion of each of the urine extracts used in the preceding experiments was set aside for a parallel series of observations with the absolute alcohol procedure. From 0.2 to 2.0 cc. of each of these 95% alcoholic urine extracts were taken to dryness over a steam bath under reduced pressure and absolute alcohol was added to the residue in amounts corresponding to those evaporated in each instance. To 0.2 cc. of each of these solutions, or to smaller amounts diluted to 0.2 cc. with absolute alcohol, were then added 0.2 cc. of a solution of m-dinitrobenzene in absolute alcohol (11.6 mg. per cc.) and 0.2 cc. 2.5N KOH in absolute alcohol. The alcoholic KOH was prepared in an iced water bath at 0°C. with constant stirring of the mixture by a motor-driven propeller. This reaction mixture was kept in a water bath at 25°C. in semi-darkness with occasional shaking. After standing for 80 minutes, the mixture was diluted with 20 cc. 95% alcohol and the color read immediately in the Evelyn photoelectric colorimeter with a No. 540 filter

Observations and conclusions. The character and magnitude of the error introduced by the aforementioned deviation from proportionality were obtained from a chart (fig. 1) on which mg. equivalents of androsterone (ordinate) were plotted against cc. of urine extract (abscissa). The following conclusions were derived from these and the unplotted data included in table 1.

(*a*) All of the curves on the plot take the form of straight lines (fig. 1).

(*b*) It is possible to restore the proportionality between mg. equivalents of androsterone and cc. of urine by subtracting a correction factor from each of the values determined for the various aliquots of urine extract used for assay. This correction factor is calculated for each urine extract by taking a reading at the intersection of the corresponding curve with the ordinate (fig. 1, table 1).

TABLE I

a) Specimen No. b) Correction factor c) Volume of extract	Volume of extracts used for assay, cc.	Mg. equivalents androsterone in volume used (uncorrected values)	Mg. equivalents androsterone in 1 cc. (uncorrected values)	Mg. equivalents androsterone in 1 cc. (corrected values)	% Difference between corrected and uncorrected values
a) 1					
b) .006					
c) Original volume 25 cc. of which 0.75 cc. was diluted to 5 cc. with 95% alcohol	0.05	0.06	1.16	1.04	10.0
	0.10	0.12	1.16	1.10	5.1
	0.15	0.17	1.14	1.10	3.4
	0.20	0.22	1.10	1.07	2.5
	0.07	0.06	0.80	0.72	10.0
a) 2	0.10	0.08	0.81	0.75	7.4
b) .006	0.15	0.12	0.77	0.73	5.1
c) 25 cc.	0.20	0.15	0.75	0.72	4.0
a) 3	0.10	0.06	0.58	0.50	13.7
b) .008	0.15	0.08	0.56	0.51	8.9
c) 25 cc.	0.20	0.11	0.54	0.50	7.4
a) 4	0.80	0.05	0.07	0.06	16.9
b) .008	1.00	0.06	0.06	0.06	12.7
c) 25 cc.	1.20	0.07	0.06	0.06	9.8
	0.40	0.05	0.12	0.09	22.5
a) 5	0.60	0.07	0.11	0.10	11.8
b) .010	0.80	0.09	0.11	0.10	12.7
c) 25 cc.	1.00	0.10	0.10	0.10	6.0
a) 6					
b) .010					
c) Original volume 25 cc. of which 1 cc. was diluted to 5 cc. with 95% alcohol	0.05	0.06	1.24	1.04	16.2
	0.10	0.17	1.12	1.06	8.6
	0.15	0.17	1.16	1.09	6.0
	0.20	0.22	1.12	1.07	4.4
	0.60	0.05	0.08	0.06	22.5
a) 7	0.80	0.06	0.08	0.06	18.2
b) .011	1.00	0.07	0.07	0.06	15.7
c) 25 cc.	1.20	0.09	0.07	0.06	14.0
	0.80	0.05	0.06	0.05	25.0
a) 8	1.20	0.07	0.06	0.05	18.6
b) .013	1.60	0.09	0.06	0.05	14.0
c) 25 cc.	2.00	0.11	0.06	0.05	12.7
	2.40	0.13	0.05	0.05	9.6
a) 9	0.10	0.07	0.71	0.58	18.3
b) .013	0.15	0.10	0.69	0.60	13.0
c) 10 cc.	0.20	0.13	0.65	0.58	10.7
a) 10	0.10	0.06	0.56	0.43	23.2
b) .013	0.15	0.08	0.53	0.44	16.9
c) 5 cc.	0.20	0.10	0.49	0.42	14.3
a) 11	0.10	0.07	0.73	0.56	23.2
b) .017	0.15	0.11	0.72	0.61	15.2
c) 10 cc.	0.20	0.13	0.67	0.59	11.9
	0.05	0.07	1.44	1.00	30.5
a) 12	0.10	0.13	1.31	1.09	16.8
b) .022	0.15	0.18	1.22	1.07	12.3
c) 25 cc.	0.20	0.23	1.16	1.05	9.4

TABLE I (*Continued*)

a) Specimen No. b) Correction factor c) Volume of extract	Volume of extracts used for assay, cc.	Mg. equivalents androsterone in volume used (uncorrected values)	Mg. equivalents androsterone in 1 cc. (uncorrected values)	Mg. equivalents androsterone in 1 cc. (corrected values)	% Difference between corrected and uncorrected values
	0.10	0.05	0.47	0.25	46.8
	0.20	0.09	0.45	0.33	26.6
a) 13	0.30	0.12	0.39	0.31	20.5
b) .022	0.40	0.15	0.37	0.32	13.5
c) 25 cc.	0.50	0.18	0.36	0.31	13.8
	0.60	0.21	0.34	0.31	8.8
	0.70	0.23	0.33	0.30	9.0
	0.20	0.05	0.23	0.12	47.8
a) 14	0.40	0.08	0.20	0.14	30.0
b) .022	0.60	0.11	0.18	0.14	22.2
c) 25 cc.	0.80	0.13	0.17	0.14	17.6
	0.80	0.06	0.07	0.39	43.4
a) 15	1.00	0.06	0.06	0.34	41.3
b) .024	1.20	0.07	0.06	0.39	33.8
c) 25 cc.	1.40	0.08	0.06	0.39	30.3
	0.15	0.05	0.32	0.16	50.0
	0.20	0.06	0.30	0.18	40.0
a) 16	0.40	0.09	0.23	0.17	26.0
b) .024	0.60	0.13	0.21	0.17	19.0
c) 25 cc.	0.80	0.16	0.20	0.17	15.0
	1.00	0.16	0.16	0.14	12.5
	0.15	0.05	0.36	0.19	47.2
a) 17	0.20	0.07	0.34	0.22	35.2
b) .025	0.40	0.11	0.27	0.21	22.2
c) 25 cc.	0.60	0.15	0.26	0.21	19.2
	0.80	0.19	0.23	0.20	13.0
	0.05	0.08	1.62	1.12	30.7
a) 18	0.10	0.14	1.40	1.15	17.8
b) .025	0.15	0.19	1.28	1.11	13.5
c) 25 cc.	0.20	0.23	1.14	1.01	11.3
	0.20	0.06	0.28	0.14	49.0
a) 19	0.40	0.08	0.19	0.12	36.8
b) .027	0.60	0.11	0.19	0.14	24.3
c) 25 cc.	0.80	0.14	0.17	0.14	18.6
	1.00	0.17	0.17	0.14	15.6
	0.05	0.05	0.90	0.34	62.2
	0.10	0.08	0.77	0.49	36.3
a) 20	0.15	0.11	0.71	0.53	25.3
b) .028	0.20	0.13	0.65	0.51	21.5
c) 25 cc.	0.30	0.18	0.60	0.50	16.6
	0.40	0.22	0.56	0.49	12.5
	0.50	0.26	0.51	0.47	7.8
	0.10	0.05	0.51	0.21	58.8
a) 21	0.15	0.07	0.48	0.28	41.6
b) .030	0.20	0.09	0.46	0.31	32.6
c) 25 cc.	0.40	0.15	0.38	0.30	21.0
	0.60	0.21	0.35	0.30	14.2
	0.80	0.27	0.35	0.29	17.1

TABLE 1 (*Continued*)

a) Specimen No. *b*) Correction factor *c*) Volume of extract	Volume of extracts used for assay, cc.	Mg. equivalents androsterone in volume used (uncorrected values)	Mg. equivalents androsterone in 1 cc. (uncorrected values)	Mg. equivalents androsterone in 1 cc. (corrected values)	% Difference between corrected and uncorrected values
	0.10	0.05	0.54	0.22	59.2
a) 22	0.15	0.08	0.53	0.32	39.6
b) .032	0.20	0.10	0.50	0.33	34.0
c) 25 cc.	0.40	0.15	0.39	0.31	20.4
	0.60	0.21	0.35	0.30	14.2
	0.80	0.25	0.32	0.28	12.5
	0.10	0.05	0.52	0.21	59.6
a) 23	0.15	0.07	0.48	0.27	43.7
b) .032	0.20	0.09	0.45	0.29	35.5
c) 25 cc.	0.40	0.13	0.33	0.25	24.2
	0.60	0.19	0.31	0.26	16.1
	0.80	0.22	0.28	0.24	14.2
	0.10	0.07	0.71	0.34	52.1
a) 24	0.15	0.10	0.69	0.44	36.2
b) .037	0.20	0.13	0.64	0.46	28.1
c) 25 cc.	0.30	0.17	0.56	0.44	21.4
	0.40	0.21	0.53	0.44	16.9
	0.50	0.24	0.48	0.41	14.5
	0.05	0.08	1.56	0.76	51.2
a) 25	0.10	0.13	1.25	0.85	32.0
b) .040	0.15	0.16	1.09	0.83	23.8
c) 5 cc.	0.20	0.20	0.99	0.79	20.2
	0.10	0.06	0.58	0.18	68.9
a) 26	0.15	0.08	0.54	0.27	50.0
b) .040	0.20	0.10	0.50	0.29	42.0
c) 25 cc.	0.40	0.15	0.37	0.27	27.0
	0.60	0.20	0.33	0.27	18.1
	0.80	0.24	0.30	0.25	16.6
	0.10	0.06	0.59	0.17	71.1
a) 27	0.15	0.08	0.55	0.27	50.9
b) .042	0.20	0.10	0.52	0.30	42.3
c) 25 cc.	0.40	0.15	0.37	0.27	27.0
	0.60	0.20	0.34	0.27	20.5
	0.80	0.25	0.32	0.27	15.6
	0.10	0.06	0.61	0.18	70.4
a) 28	0.15	0.09	0.59	0.30	49.1
b) .043	0.20	0.11	0.54	0.32	40.7
c) 25 cc.	0.40	0.16	0.39	0.28	28.2
	0.60	0.22	0.36	0.29	19.4
	0.80	0.27	0.33	0.28	15.1
	0.10	0.07	0.69	0.22	68.5
a) 29	0.15	0.10	0.65	0.33	49.0
b) .047	0.20	0.12	0.58	0.35	39.6
c) 25 cc.	0.40	0.18	0.44	0.32	27.2
	0.60	0.24	0.40	0.32	20.0
	0.80	0.29	0.37	0.31	16.2

(*c*) The correction factors in 29 such determinations ranged from 0.006 to 0.047 with a mean of 0.023 mg. equivalents of androsterone (table 1).

(*d*) A correction factor must be determined for each urine extract since it is not constant for different specimens of urine from the same patient (table 1).

(e) Our colorimetric determinations have been made routinely with 0.2 cc. of each urine extract which contains the 24-hour ketosteroid excretion diluted to 25 cc. with 95% alcohol. Only a negligible error of approximately 10% in the determination of ketosteroid content is encountered with this procedure in the examination of urines having a relatively large amount of these substances (in virilistic patients with and without corticoadrenal tumor); whereas the assay of urines from normal individuals, who excrete smaller amounts of ketosteroids, gives results which are an average of 39% and in some instances are as much as 70% higher than the probable ketosteroid

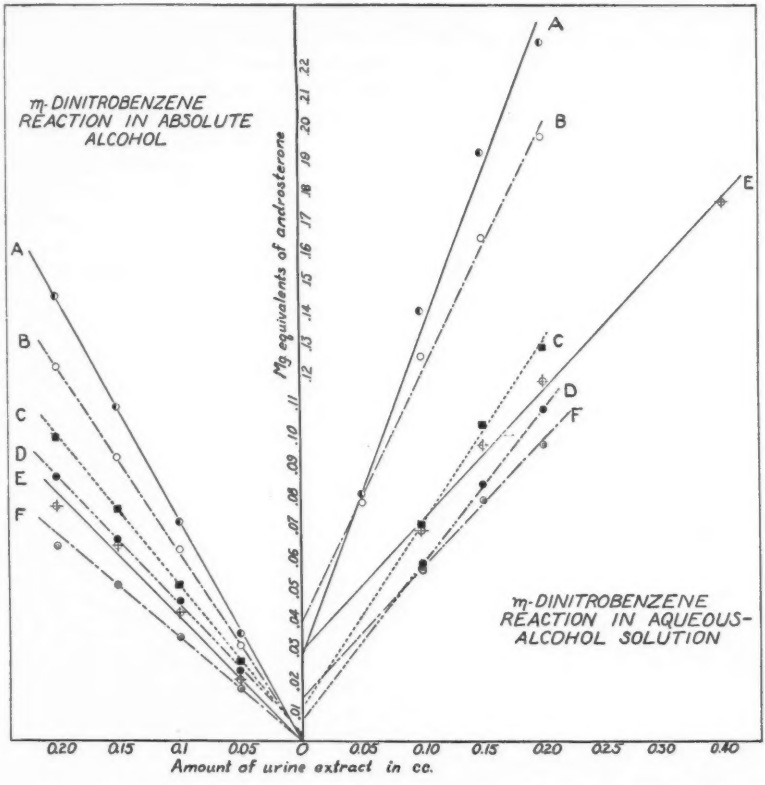

Fig. 1. Effect of water on accuracy of m-dinitrobenzene reaction in the quantitative assay of urinary ketosteroids. A, specimen 18; B, specimen 25; C, specimen 9; D, specimen 3; E, specimen 29; F, specimen 10.

content of such urines. If 0.2 cc. of such an extract or less is used for assay the percentage error in the determination is of significant proportions; if more than 0.2 cc. is taken for assay, the error is of far less consequence. In the latter instance the error may be decreased from the average of 39% to approximately 15% by increasing the amount of urine extract used for each determination from 0.2 cc. to 1.0 cc. A similar purpose can be accomplished more conveniently by using 0.2 cc. of a urine extract dissolved in 5 cc. instead of 25 cc. alcohol. In actual practice it has been found that the conclusions reached by Callow, Callow and Emmens (4) with their modification

of the Wu and Chou absolute alcohol technic are the same as those of Friedgood and Whidden (15) who studied similar groups of patients with the aqueous alcohol method. The range of ketosteroid excretion in each of these groups of patients is large enough so that the use of either the aqueous alcohol or absolute alcohol methods results in essentially the same general correlations between the ketosteroid content of urine extracts and the clinical condition of the individuals being studied.

The probable cause for the deviation from proportionality. The lack of proportionality is not due to the interfering action of colored pigments of urine extracts for most of them were decolorized with Norite A prior to assay in order to rule out this possibility. The adsorption by Norite A of a portion of the ketosteroid content of the urine was of little consequence in these experiments which were devised essentially for the purpose of studying the effect of water on the m-dinitrobenzene reaction in a pigment-free urine extract. That the presence of water in the reaction mixture seemed to be the determining factor was proved by the parallel series of experiments on extracts which were assayed by the m-dinitrobenzene aqueous alcohol method and by the Wu and Chou-Callow technic as adapted for the Evelyn photoelectric colorimeter by Langstroth and Talbot (7). The necessity for an initial correction factor was eliminated in almost all of the experiments run by the latter method. Those few instances in which an exact proportionality was not obtained may be attributed to experimental error. The precise means by which water alters the prog-

TABLE 2

a) Specimen No. *b*) Volume of extract	Volume of extract used for assay, cc.	Mg. equivalents androsterone in volume used	Mg. equivalents androsterone in 1 cc.
a) 25 *b* 5 cc.	0.05	0.03	0.62
	0.10	0.06	0.63
	0.15	0.09	0.62
	0.20	0.12	0.61
a) 20 *b*) 25 cc.	0.05	0.03	0.52
	0.10	0.05	0.52
	0.15	0.08	0.52
	0.20	0.10	0.52
a) 26 *b*) 25 cc.	0.05	0.01	0.27
	0.10	0.03	0.26
	0.15	0.04	0.29
	0.20	0.05	0.27
a) 12 *b*) 25 cc.	0.05	0.06	1.10
	0.10	0.09	0.93
	0.15	0.14	0.92
	0.20	0.18	0.92
a) 27 *b*) 25 cc.	0.05	0.12	0.31
	0.10	0.03	0.31
	0.15	0.05	0.31
	0.20	0.06	0.30
a) 29 *b*) 25 cc.	0.05	0.02	0.40
	0.10	0.04	0.42
	0.15	0.06	0.43
	0.20	0.08	0.39
a) 24 *b*) 25 cc.	0.05	0.02	0.41
	0.10	0.04	0.44
	0.15	0.07	0.47
	0.20	0.09	0.46

TABLE 2 (*Continued*)

a) Specimen No. b) Volume of extract	Volume of extract used for assay, cc.	Mg. equivalents androsterone in volume used	Mg. equivalents androsterone in 1 cc.
	0.05	0.04	0.69
a) 18	0.10	0.07	0.72
b) 25 cc.	0.15	0.11	0.73
	0.20	0.15	0.73
	0.05	0.02	0.37
a) 10	0.10	0.03	0.34
b) 5 cc.	0.15	0.05	0.34
	0.20	0.06	0.32
	0.05	0.02	0.39
a) 22	0.10	0.04	0.37
b) 25 cc.	0.15	0.06	0.39
	0.20	0.08	0.39
	0.05	0.02	0.31
a) 13	0.10	0.03	0.32
b 25 cc.	0.15	0.05	0.33
	0.20	0.06	0.31
	0.05	0.04	0.69
a) 28	0.10	0.07	0.67
b) 25 cc.	0.15	0.10	0.65
	0.20	0.13	0.63
	0.05	0.03	0.52
a) 9	0.10	0.05	0.51
b) 10 cc.	0.15	0.08	0.51
	0.20	0.10	0.50
	0.05	0.01	0.20
a) 17	0.10	0.02	0.20
b) 25 cc.	0.15	0.04	0.24
	0.20	0.05	0.23
	0.05	0.02	0.45
a) 3	0.10	0.05	0.46
b) 25 cc.	0.15	0.07	0.44
	0.20	0.09	0.44

ress of the chemical reaction in a urine extract is still obscure, but experiments are now being made to ascertain this point.

The corrected values of the aqueous alcohol procedure are generally in close agreement with the values obtained by the absolute alcohol technic. They differ significantly in only a few instances, some of which may be attributed to experimental error. The correction factors presumably make allowance for the difference in the color producing properties of such androgens as androsterone and dehydroisoandrosterone. Furthermore, the determinations by both methods were not always run at the same time and changes may have occurred in the concentration of ketosteroids due to the evaporation of alcohol. We have repeatedly found, moreover, that there is an apparent increase in the ketosteroid content of urine extracts when assayed by the Zimmermann reaction after the storage of these substances in 95% alcohol. Because of this progressive change it is essential to assay urine extracts as soon as possible after they are prepared.

The choice of a method for routine clinical use. On the basis of a fairly extensive series of observations on corresponding groups of patients (15) it is clear that for practical use, the purpose of which is to correlate the ketosteroid content of urine with the

clinical condition of the patient, the results obtained by either method are equally useful even if the initial correction factor is omitted from the calculations in the aqueous-alcohol technic. From a chemical viewpoint, however, it is recommended that one of the acceptable modifications of the Wu and Chou-Callow procedure be adopted universally for the determination of the ketosteroid content of urine extracts. In spite of the somewhat lengthy manipulations involved in the preparation of the reagents in absolute alcohol and the increase in the intensity of the color of the blank solution, this method offers a means of eliminating an important source of avoidable error. The choice of this method does not imply that the values obtained with it give the absolute ketosteroid content of a urine extract. Results obtained with the polarograph by Fieser and his coworkers Wolfe and Hershberg (16) indicate that the actual ketosteroid content of a urine extract is somewhat less than that which is observed by conducting the assay with m-dinitrobenzene reaction in absolute alcohol.

SUMMARY

The m-dinitrobenzene reaction for the quantitative determination of ketosteroids is currently being carried out in the form of a number of modifications of the original Zimmermann procedure which differ fundamentally in only one respect, viz., the water content of the reaction mixture. It was found in a previous investigation that the presence of water in a reaction mixture containing crystalline androsterone and dehydroisoandrosterone interferes to some extent with the accuracy of its assay. A significant error has now been detected in the assay of urine extracts for their ketosteroid content by the same method, e.g., doubling the amount of urine extract used for assay by the aqueous KOH method does not result in double the intensity of color which would be expected on the basis of Beer's law from a 2-fold increase in the amount of ketosteroids. This error is reduced to minor proportions if 1.0 cc. instead of 0.2 cc. of a urine extract is used for each colorimetric determination. The deviation from proportionality is obviated by the use of the Wu and Chou-Callow absolute alcohol technic.

Although a correction factor can be calculated for each urine extract if the aqueous KOH method is used, it is urged that the absolute alcohol procedure be adopted universally for the determination of the ketosteroid content of urine extracts in order to standardize this technic and make the results reported by different investigators more readily comparable.

REFERENCES

1. Zimmermann, W.: *Ztschr. f. Physiol. Chem.* 233: 257. 1935.
2. Zimmermann, W.: *Ztschr. f. Physiol. Chem.* 245: 47. 1936.
3. Wu, H., and C. Y. Chou: *Chinese J. Physiol.* 11: 413. 1937.
4. Callow, N. H., R. K. Calow and C. W. Emmens: *Biochem. J.* 32: 1312. 1938.
5. Oesting, R. B.: *Proc. Soc. Exper. Biol. & Med.* 36: 524. 1937.
6. Oesting, R. B., and B. Webster: *Endocrinology* 22: 307. 1938.
7. Langstroth, G. O., and N. B. Talbot: *J. Biol. Chem.* 129: 759. 1939.
8. Friedgood, H. B., and H. L. Whidden: *New England J. Med.* 220: 736. 1939.
9. Friedgood, H. B., and H. L. Whidden: *Endocrinology* 27: 258. 1940.
10. Friedgood, H. B., and H. L. Whidden: *Endocrinology* 25: 919. 1939.
11. Friedgood, H. B., and H. L. Whidden: *Endocrinology* 28: 237. 1941.
12. Friedgood, H. B., and H. L. Whidden: *Endocrinology* 27: 241. 1940.
13. Friedgod, H. B., and H. L. Whidden: *Endocrinology* 27: 249. 1940.
14. Langstroth, G. O., and N. B. Talbot: *J. Biol. Chem.* 129: 759. 1939.
15. Friedgood, H. B., and H. L. Whidden: Urinary Ketosteroids in Various Endocrinopathies. In preparation.
16. Wolfe, J. K., E. B. Hershberg and L. F. Fieser: *J. Biol. Chem.* (in press).

SOME COMPARATIVE EFFECTS OF TESTOSTERONE DERIVATIVES IN THE MONKEY[1]

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IN A RECENT PUBLICATION we compared the effects (1) in the immature castrate macaque following subcutaneous injections of testosterone propionate (perandren) with those following implantations of crystalline testosterone. It was found under the experimental conditions used that testosterone in the form of crystalline pellets produced a greater relative increase in weight in the accessory genitalia tissue. In those experiments the increase in weight of the accessory genitalia was used as a criterion to compare the effectiveness of administering testosterone by the two methods. It was also noted that the histologic changes following pellet implantation were identical with those following injections. Our experiments indicated that in the primate implantations of testosterone in the form of crystals was a more efficient and physiological method than that of injecting testosterone propionate in oil at intervals of 2 or 3 times per week. The important clinical implications of these observations in the monkey were discussed. One of us (S. A. V.) and Dr. John Eager Howard, in 1937, began to study the effects of subcutaneous implantation of testosterone crystals in man (2–5) and in a forthcoming publication further observations on the effect of implantation in man, not only of testosterone, but of various esters, will be presented.

It has been shown by Miescher (6, 7) and his coworkers in their intensive study of various esters and substitution products of testosterone, that esters, such as testosterone 17-propionate or a diester, 3,17-propionate, are more effective in the rat than in the capon. He also showed that they have a much more intense and prolonged effect when given in single instead of multiple doses. It was mainly for this reason that the propionate was selected for clinical use in man. It is probable, although not definitely settled, that the testosterone molecule is the active principle of the ester compounds rather than the entire testosterone ester molecule itself, and the enhanced action of the ester is due to delayed absorption resulting in a more constant rather than a sudden flow from the oily deposit into the blood stream. The propionic acid ester, however, does not yield, under all conditions, a more pronounced effect. As we have shown that the daily absorption of 2 to 5 mg. of testosterone in either human beings or macaque monkeys from an implanted pellet is able to duplicate the effect of a greater amount of the propionate injected intramuscularly (4). While carrying on the experiments in the macaques to compare the relative effectiveness of testosterone pellets, it seemed desirable to implant pellets of an ester, a diester and an alkyl-substituted compound and compare their effects with those of non-esterified testosterone. Our observations on such effects are herewith presented.

Received for publication August 8, 1940.
[1] This work was supported by a fellowship from the Lalor Foundation and a grant from the Ciba Pharmaceutical Products, Inc. who also supplied the androgens.

Three immature macaque monkeys of similar age and weight as used in our previous experiments (1) were castrated in April, 1939, and treated as follows: In animal 1 a pellet of testosterone 17-methyl weighing 333 mg. was implanted subcutaneously along the lumbo-dorsal fascia. It was removed at the end of 66 days, at which time 133 mg. had been absorbed, or approximately 2 mg. per day. Another pellet was immediately implanted weighing 348 mg. and removed 22 days later. During this period 41 mg., or an average of 1.9 mg. was absorbed per day. The animal was killed at the end of 88 days. Figure 1 shows the size of the prostate, both cranial and caudal lobes and seminal vesicles, as compared to those of an untreated castrate macaque of approximately similar age and weight.

Figures 2, 3 and 4 show the relative effectiveness following the implantation of

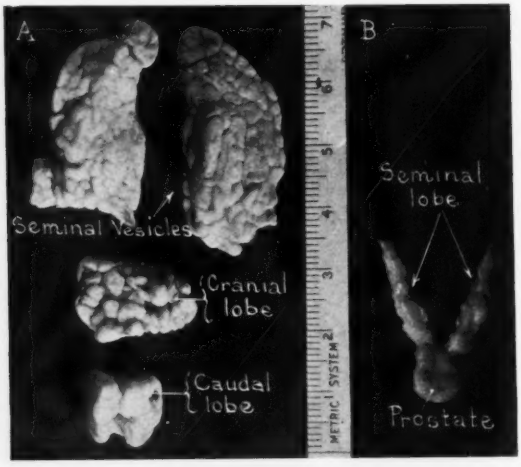

Fig. 1. A. The accessory genitalia of the animal which received methyl testosterone in the form of pellets planted subcutaneously. The caudal and cranial lobe have been dissected out individually and also the seminal vesicles. B. This prostate and seminal vesicle are from an approximately similar age castrate control animal. For purposes of comparison both photographs were taken at the same magnification as indicated by the metric rule.

methyl testosterone when compared with that of pellets of testosterone, testosterone 17-propionate and testosterone 3,17-propionate. It can be noted that methyl testosterone has practically the same effectiveness as pure testosterone when administered in the form of an implanted pellet. The molecular weight of methyl testosterone compared to the others is such that practically 95% of the molecule is testosterone. This experiment with methyl testosterone was carried out because Miescher and Tschopp (8) have shown the remarkable effect of methyl testosterone over testosterone or its various esters when given orally. They found it a number of times more effective than the propionate and definitely more effective than testosterone itself when administered orally, indicating that the union of a methyl group at carbon atom 17 withstands the influence of intestinal enzymes and the destructive process in the liver, etc. better than esterification. The possibility that the molecule of methyl testosterone is active itself when administered by mouth cannot be excluded with certainty. The possibility, however, that the molecule is active itself is relatively small because when methyl testosterone is given parenterally in oily solution it has been shown to be slightly more active than testosterone but definitely less active than pro-

pionate. Our experiments in the monkey do not indicate any quantitative or qualitative difference as far as the 17-methyl is concerned when compared to crystalline testosterone by our method of administration.

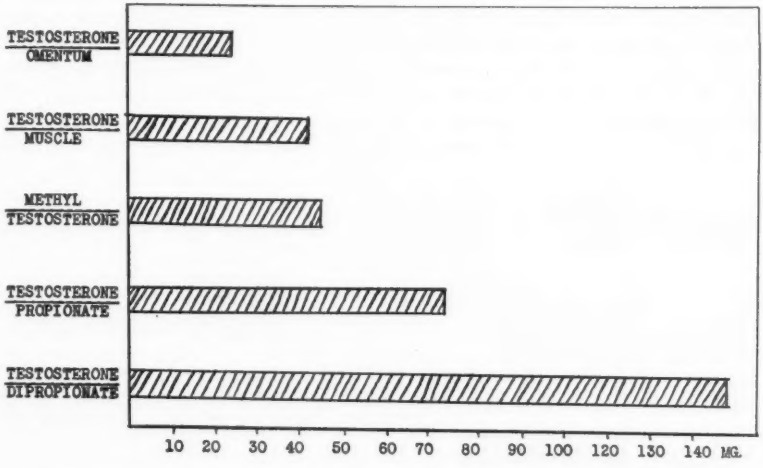
TESTOSTERONE OMENTUM
TESTOSTERONE MUSCLE
METHYL TESTOSTERONE
TESTOSTERONE PROPIONATE
TESTOSTERONE DIPROPIONATE
10 20 30 40 50 60 70 80 90 100 120 130 140 MG.

Fig. 2. The weight of the accessory genitalia (prostate plus both seminal vesicles) per mg. of hormone absorbed. The striking effect of testosterone dipropionate is evident as each mg. absorbed resulted in approximately 150 mg. of accessory genitalia.

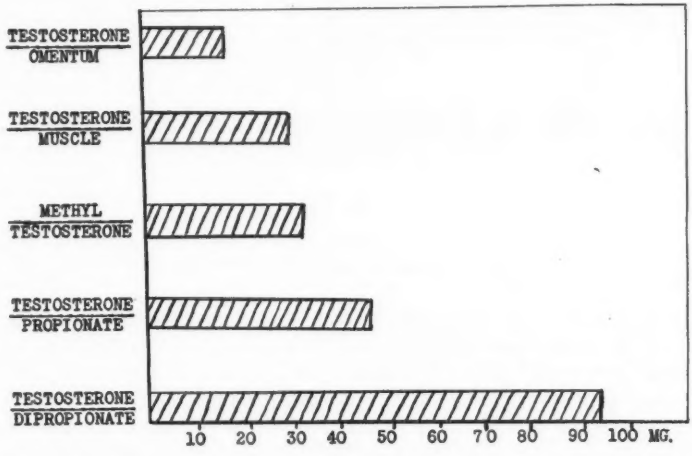

Fig. 3. The weight of seminal vesicle tissue per mg. of hormone absorbed and again the striking increase in efficiency of the dipropionate is evident.

An immature macaque of similar age and weight was castrated in April, 1939 and one month later a pellet of testosterone 17-propionate weighing 386 mg. was implanted subcutaneously on the lumbo-dorsal fascia. At the end of 42 days, at which time the animal was killed, it was found that 75 mg. had been absorbed, an average

of 1.79 mg. per day. Figures 2, 3 and 4 show the weight in accessory genitalia per mg. of hormone absorbed. In figure 1 it can be seen that there resulted 13.3 mg. of cranial prostatic lobe and 11.7 mg. of caudal lobe per mg. of absorbed testosterone propionate. Figure 3 shows the effect on the seminal vesicles in which case there resulted 46.8 mg. of seminal vesical tissue per mg. of absorbed testosterone propionate. In figure 4 the entire accessory genitalia, which includes both lobes of the prostate and the seminal vesicles, are calculated as weight of this total tissue per mg. of absorbed hormone. In the case of the propionate there were 71.9 mg. of total accessory genitalia tissue per mg. of absorbed hormone.

A third immature castrate macaque of similar age and weight was treated in

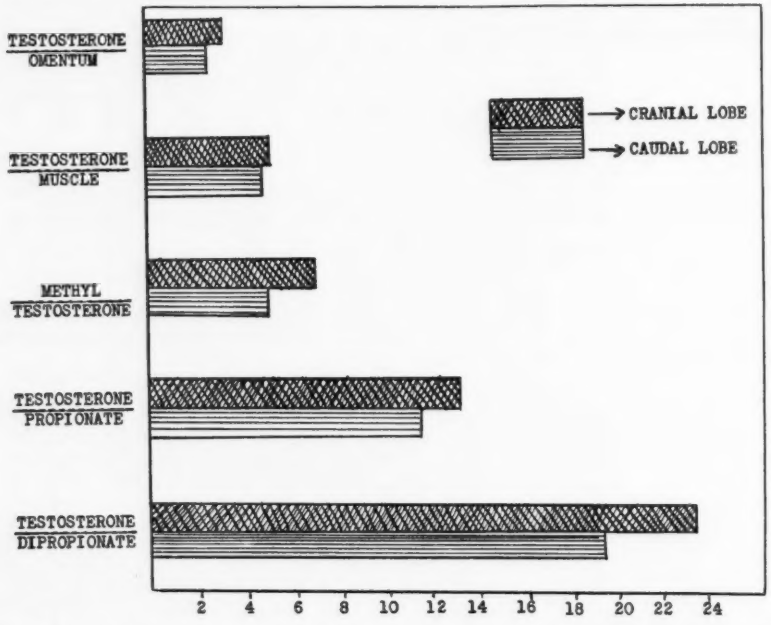

Fig. 4. The weight of prostatic tissue, both cranial and caudal lobe per mg. of hormone absorbed. The increase in efficiency of the dipropionate over the other compounds is striking and is the same in either caudal or cranial lobe.

March, 1939 by the implantation of a pellet of testosterone 3,17-dipropionate weighing 214 mg. This was removed at the end of 71 days and a new pellet weighing 299 mg. inserted for a further period of 19 days, at which time the animal was killed. The average daily absorption during this period was 0.3 mg. per day. Figures 2, 3 and 4 show the remarkable effectiveness of testosterone 3,17-dipropionate in producing increased weight of the accessory genitalia. If we compare the molecular weight of this ester with that of testosterone we find that only slightly more than two-thirds of the molecule is testosterone.

MOLECULAR WEIGHTS

Testosterone	288
Testosterone propionate	344
Testosterone dipropionate	410
Methyl testosterone	302

When based on comparative molecular weights, the efficiency of the dipropionate of pure testosterone would be even more striking. As stated before the increased effect of enol di-esters may be merely one of delayed absorption. In the use of these compounds by implantation, two factors to enhance the effectiveness may be at work. The esterified molecule itself and the fact that it is administered in the form of a hard, compact pellet which gives off only a few mg. or a fraction thereof a day at a fairly even though slightly decreasing rate.

Figure 5 shows the average daily absorption of testosterone compared with its 3 substituted products used in this experiment. It will be noted that testosterone im-

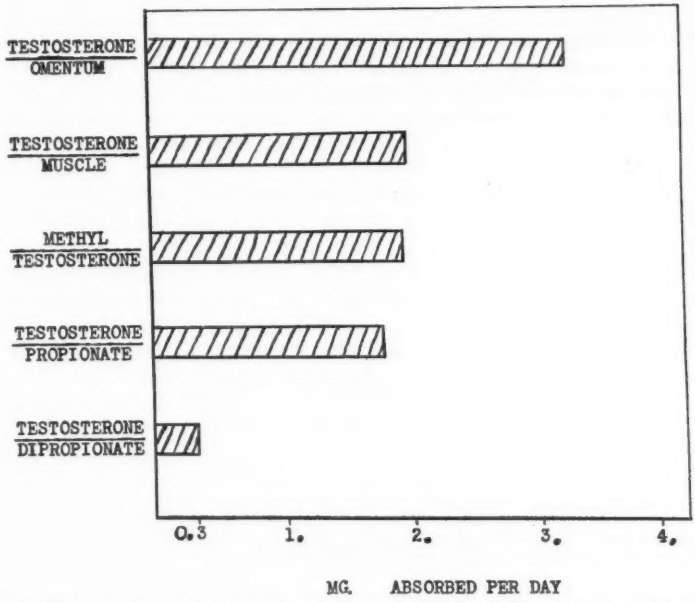

Fig. 5. The average daily absorption in mg. from several pellets of the various compounds. Only 0.3 mg. a day of testosterone dipropionate was absorbed compared to an average of over 3 mg. a day of pure testosterone when implanted in the omentum.

planted in the omentum is absorbed faster than approximately the same size pellet implanted in muscle, and as shown in previous experiments (1) with correspondingly less effect. It is probable that there is less tendency for a relatively avascular fibrous tissue capsule to form around the pellet in the omentum and local conditions are more favorable for rapid absorption.

DISCUSSION

The observations in these experiments indicate that the esters of testosterone which are known to be more insoluble are also absorbed more slowly when administered in the form of hard, compact pellets. Under the experimental conditions used the di-ester, testosterone 3,17-dipropionate, is far more efficient than the 17-monopropionate of testosterone or methylated testosterone. The relative increase in efficiency of the esters is the same whether the cranial or caudal lobe of the prostate is used for comparison, or whether it be the seminal vesicles. As indicated in our previous experiments, the increase in weight in seminal vesicle tissue was somewhat greater

in proportion than the increase in weight of the prostate. The changes following development of the seminal vesicles found on microscopic examination were the same as those found in normal animals in previous experiments. These results indicate that pellets of the esters of testosterone are absorbed more slowly than pellets of pure testosterone, and if sufficiently large would be more economical, efficient and last a much longer period before the necessity of a new implantation.

Although these observations are single and isolated, the data obtained indicates that the use of esters and di-esters of testosterone in the form of large pellets might probably be more efficient in human beings than testosterone itself.

SUMMARY

The effectiveness of pellets of testosterone 17-methyl, of testosterone 17-propionate (perandren) and 3,17-propionate (dipropionate) have been compared with testosterone in castrated immature macaque monkeys.

The increase in the weight of the cranial and caudal lobes of the prostate and seminal vesicles per amount of hormone absorbed from the pellets was used as a criterion of effectiveness.

The relative effectiveness of the compounds decrease in the following order: testosterone 3,17-dipropionate, testosterone 17-propionate, testosterone 17-methyl and testosterone.

The average daily absorption from pellets of this material decreases in the following order: testosterone, testosterone 17-methyl, testosterone 17-propionate, and testosterone 3,17-propionate. The 3,17-propionate (testosterone dipropionate) is absorbed at about $\frac{1}{6}$ the rate of testosterone and is about $3\frac{1}{2}$ times more effective.

REFERENCES

1. VEST, S. A., J. E. DREW AND O. R. LANGWORTHY: *Endocrinology*: 7: 455. 1940.
2. VEST, S. A., AND J. E. HOWARD: *J. Urol.* 40: 154. 1938.
3. HOWARD, J. E., AND S. A. VEST: *Am. J. Med. Sci.* 198: 823. 1939.
4. VEST, S. A., AND J. E. HOWARD: *J.A.M.A.* 113: 1869. 1939.
5. RENNIE, T. A. C., S. A. VEST AND J. E. HOWARD: *Southern M. J.* 32: 1004. 1939.
6. MIESCHER, K., W. H. FISCHER AND E. TSCHOPP: *Nature* 140: 726. 1939.
7. MIESCHER, K., H. KÄGI, C. SCHOLZ, A. WETTSTEIN AND E. TSCHOPP: *Biochem. Ztsch.* 67: 268. 1937.
8. MIESCHER, K., AND E. TSCHOPP: *Schweizer. Med. Wchnschr.* 68: 1258. 1938.

Addendum. Since making the above study, the authors have had the occasion to investigate the use of pellets of the dipropionate of testosterone in castrate males. Due probably to the slow absorption rate, there has been very little discernible effect after implantation of this androgen. For an example, in one castrate 6 pellets averaging 200 mg. were implanted for 140 days. The average absorption was 1 mg. per day from which there was only slight subjective but no objective effects. It is apparent that the amount necessary to produce growth of the monkey prostate is of considerably lower magnitude than that necessary to produce changes in man.

EXPERIMENTAL STUDIES ON THE ACTIVITY AND TOXICITY OF STILBESTROL

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THE CHEMICAL NAME for a new synthetic estrogenic substance frequently referred to as diethylstilbestrol is 4′,4′-dihydroxy-a-b-diethylstilbene. Discovered by Dodds and coworkers (1) in 1937, it immediately aroused keen interest and in the few years following his discovery a large number of publications have appeared discussing the specific and toxic effects of this drug in animals and man. In a preliminary report the Council on Pharmacy and Chemistry of the American Medical Association (2) on the basis of certain investigations (3, 4, 5) recommended that the outcome of further investigations should be awaited before official approval of this drug and its inclusion in our medical armamentarium.

We have been interested in the various phases of activity and toxicity of stilbestrol for the past year and have conducted numerous animal experiments which in part tend to confirm observations already made, in part to add new data to our knowledge. Our experiments were performed on immature, adult and castrated mice and rats and are grouped in two chapters: those experiments intended to bring out additional factors concerning the estrogenic activity of stilbestrol, in which we used estrone injected in similar doses for comparison; and those which contrast the toxic effect of this synthetic estrogenic substance with similar doses of natural hormone. Each chapter is introduced by a brief summary of the pertinent literature. Because of the various methods used in each of our experiments we shall mention them briefly in the discussion of our results.

A. Experiments on the Specific and Nonspecific Effects of Stilbestrol

Numerous reports on the estrogenic effect of this substance in premature and castrated animals have appeared. Dodds (6) himself with numerous coworkers showed that in castrated rats as little as 1 gamma of the drug injected subcutaneously produced definite growth of the atrophic uterus. The duration of estrus using a 1 gamma dose was from 3 to 5 days. In castrated mice the estrus producing dose according to Kreitmair and Sieckman (7) was 0.18 gamma for estrostilbene 0.075 gamma for the diacetate, 0.25 gamma for the dipropionate, and 0.1 gamma for estrone.

The relationship between the oral and the parenteral doses is still a matter for dispute and conflicting data have been published (8, 9) Varangot (10) found that stilbestrol given parenterally to castrated rats produced an activity 6 times greater than estradiol benzoate, a figure which seems too high as compared with other authors. In the careful experiments of Sondern (11) 5 times as much stilbestrol was required orally to produce estrus in 50% of adult castrated mice in contrast to estrone, of which one hundred times the parenteral dose, and estradiol, of which five hundred times the parenteral dose is necessary.

Experiments on infantile animals (12) showed that the drug had approximately the same

Received for publication August 23, 1940.
Aided by a grant from the Comly Foundation for Medical Research.

effect as estradiol and twice the effect of estrone. Grab emphasized that esters of stilbene are much more powerful than the esters of estrone. The estrogenic effect of stilbestrol was also demonstrated for monkeys (13) for cats (14) for guinea pigs and rabbits (6). Its sensitizing actions of the uterus to progestin was proven by Leighty and Wick, and Parkes, Dodds and Noble (15) showed that oral administration inhibited implantation and interrupted established pregnancy. The sensitivity of the uterus to pitocin, which is lost about three months after castration, is not as effectively restituted by stilbestrol as by estrone (16). The stimulating effect of stilbestrol on the mammary glands of guinea pigs was less pronounced than that of the natural sex hormone (6). Doses of 3 mg. stilbestrol stopped lactation in rats, and 1 gm. of crystalline substance implanted subcutaneously into a dairy shorthorn cow in advanced lactation produced a more prolonged increase in fat and non-fatty solids similar to the effect of estrone (17). Sexual libido apparently was increased in castrated cats (Gros). If injected into embryos a similar feminizing action on the glands and gonadal ducts of genetic males could be demonstrated as with estrone (18), and Wolff (19) succeeded in the production of experimental intersexes by injecting doses of 0.01 to 0.05 mg. into chicken embryos on the fourth day of incubation.

Numerous experiments have been made on the effect of stilbestrol on the pituitary of animals. Noble (20, 21) injected the substance or implanted crystals into the subcutaneous tissue of the abdomen of rats. Daily doses of 10 mg. for 20 days caused a marked decrease of the testicles and ovaries and an increase in the adrenals, which was interpreted as the result of specific depression of the gonadotropic activity of the pituitary. It also could be shown that the body growth was inhibited for a prolonged period, and assay of the pituitary showed an absence of gonadotropic hormones. The effect on the castrate pituitary was studied by Jacobsen and Grab (22). Daily treatment of castrated animals with 5 to 100 gamma of stilbestrol produced a disappearance of vacuoles in the castration cells. It was also possible to produce estrin overdose phenomena with the production of mitoses and an increase in the chromophobe cells. Mellish, Baer and Macias (23) confirmed Noble to a large extent. They also observed an inhibition of body growth and a reduction of gonadotropic potency of the pituitary which was more marked in the female than in the male.

Other effects of this substance not intimately connected with the pituitary-gonadal mechanism may be mentioned briefly. Sherwood (24) showed that stilbestrol was not as effective as amniotin in the reduction of the basal metabolism in 16 ovariectomized and thyroidectomized rats which had been fed desiccated thyroid. Cobet and coworkers (25) showed that similar to the female sex hormone stilbestrol protected the tails of male and female rats against the necrotizing effect of single doses of ergotamin. Dawson and Robson (26) showed that stilbestrol in a concentration of 1:1,000,000 produced a similar effect on the small intestines of the rabbit as estradiol and progesterone in concentration of 1:200,000. Intravenous injection of 10 to 20 mg. of the drug produced only a small effect on the blood pressure and the intestines and uterine movements in situ. Hoskins (27) observed a constant augmentation in spontaneous activity in mature or senescent male rats following the injection of the drug. It was accompanied by weight loss ranging from 16 to 30%. Zondek (28) reported the appearance of a lipemia and calcemia in the cock treated with this drug.

Essential differences between the natural sex hormone and diethylstilbestrol have been reported by Mühlbock (29), who was unable to produce a complete suppression of growth activity in the comb of a capon stimulated by testosterone. Duyvene de Wit and Bretschneider (30) claimed that its addition to water did not produce a growth of the ovipostor of the female bitterling as demonstrable with the natural follicle hormone.

Our experiments concerning the effectivity of stilbestrol were conducted on infantile and castrated animals in which we have attempted to produce and maintain estrus in the genital organs as well as to influence castration changes of the pituitary by immediate administration of stilbestrol. All experimental results were compared with untreated animals and those which received an equal quantity of estrone.

Experiment 1. The effect of stilbestrol on the growth of the immature mouse uterus was studied in this experiment. Stilbestrol and estrone were injected in

amounts ranging from 0.01γ to 200γ divided in 2 daily doses at 24-hour intervals to immature mice weighing between 6 and 7 gm. The animals were killed on the third day (48 hours after the first injection) and the appearance of the vulva, the vaginal smear, and the weight of the uterus were studied. The graph representing the average weights of the uteri of 10 animals of each group is pictured in figure 1. It can be noted that equal doses of estrone and stilbestrol have about a similar effect on the growth of the infantile mouse uterus until a certain concentration is reached; then, probably due to the toxic effect of stilbestrol, the size of the uterus declines sharply in the animals injected with the synthetic estrogen, while the uteri of the mice injected with estrone remain on the same high level. No differences were noted in the appearance of the vaginal opening in both groups, which became apparent at the dose level of 0.1γ. The vaginal smear showed the typical mucification reaction (31) at dose levels

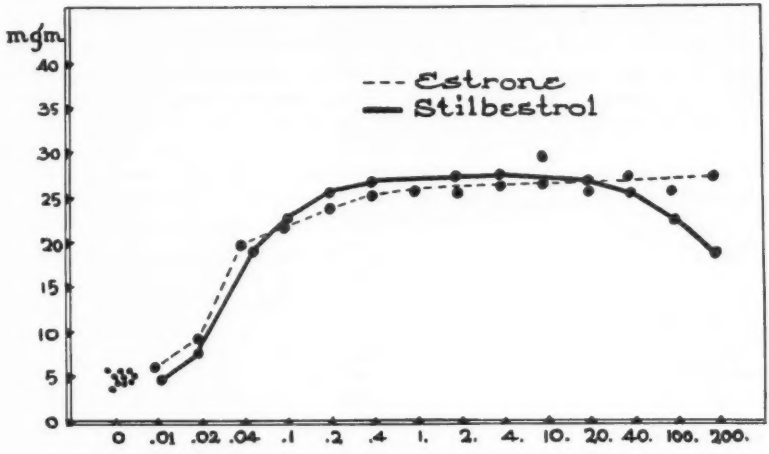

Fig. 1. Weight curve of the uteri of immature mice injected with stilbestrol and theelin.

of 0.04 to 0.2γ, which began to change into fully developed estrous smears with cornified epithelium at the 0.1 level. The relationship between uterus weight and total weight of the animals demonstrated the same depressing effect of high doses of stilbestrol on the growth of the infantile mouse uterus although not as distinctly as the values of average uterine weights, probably due to reduction of the total weight of the animals by the drug.

Experiment 2. This represents a study of the effect of equal doses of stilbestrol and estrone on the weights of the sex organs and other endocrine glands in normal and castrated rats. The estrogenic substances were injected in the amount of 1 mg. 3 times a week into adult female and male rats. At the end of one month the animals were killed and the weights of the uterus, prostate, gonads, pituitary and adrenal glands determined. Table 1 demonstrates that the increase of uterine weight was more pronounced in the animals treated with stilbestrol. In a similar way the increase of weights of the pituitary and adrenal in male and female animals was much more outstanding in the animals treated with stilbestrol, while no essential difference was present in the influence of both drugs on the weights of the ovaries and prostate.

TABLE 1. EFFECTS OF STILBESTROL IN ADULT ANIMALS

Type of animal	Uterus wt.	Prostate wt.	Ovaries wt.	Testis wt.	Pituitary wt.	Adrenal wt.
	mg.	mg.	mg.	mg.	mg.	mg.
Female rats	506		39.2		10.6	28.5
Treated with stilbestrol	809		36.4		20.5	54.8
Treated with theelin	676		34.3		16.3	46.4
Male rats		256		2413	8.9	21.9
Treated with stilbestrol		124		341	13.5	55.2
Treated with theelin		110		1240	11.6	42.3

The adrenal gland in the animals treated with stilbestrol had a brown-red color and soft consistency. The pituitaries appeared more hyperemic.

A group of rats was castrated and for one month injected 3 times a week with 0.1 mg. of stilbestrol and estrone. The increase in the weights of the pituitary and adrenal glands was more marked than in the normal adult animal (table 2). The uterine weights were approximately the same as in table 1, although the uteri of the castrated female controls were reduced 100%. The reduction in the size of the prostate was below the already very noticeable castration atrophy.

TABLE 2. EFFECTS OF STILBESTROL IN CASTRATE ANIMALS

Type of animal	Uterus wt.	Prostate wt.	Pituitary wt.	Adrenal wt.
	mg.	mg.	mg.	mg.
Females	235		10.2	60.3
Treated with stilbestrol	883		30.5	103.7
Treated with theelin	799		21.0	81.1
Males		93	13.3	49.6
Treated with stilbestrol		50.6	19.7	72.3
Treated with theelin		62.3	13.7	54.9

Experiment 3. Because of our chief interest in the therapeutic application of stilbestrol for the treatment of menstrual disorders we became much interested in the effect of stilbestrol on the state of function of the pituitary. Using the technic of Wolfe (32) we therefore made cell counts of the acidophil, basophil, and chromophobe cells of the pituitaries of animals treated with stilbestrol and compared them with the pituitaries of animals which had received the same amount of estrone. Table 3 shows the decrease in acidophil and basophil cells in both experimental groups. The decrease was more pronounced in the females treated with stilbestrol and in the males treated with estrone. Repeating the same experiment in castrate animals one month following

TABLE 3. THE EFFECTS OF STILBESTROL ON THE PITUITARY

Normal animals	Mean wt. pituitary	Acidophil cells	Basophil cells	Chromophobe cells
	mg.	%	%	%
Females	10.6	26.2	6.3	67.5
Treated with stilbestrol	20.5	14.9	2.1	83.0
Treated with theelin	16.3	18.0	3.3	78.7
Males	8.9	40.8	7.0	52.2
Treated with stilbestrol	13.5	35.7	4.8	59.5
Treated with theelin	11.6	29.6	1.7	68.7

TABLE 4. THE EFFECTS OF STILBESTROL ON THE PITUITARY OF CASTRATE ANIMALS

Castrated animals	Mean wt. pituitary	Acidophil cells	Basophil cells	Castration cells	Chromophobe cells
	mg.	%	%	%	%
Female rats	10.2	31.5	13.4	3.2	51.9
Treated with stilbestrol	30.5	20.6	5.1	0.8	73.5
Treated with theelin	21.0	23.0	1.8	0	75.2
Males	13.3	46.1	10.6	4.3	39.0
Treated with stilbestrol	19.7	32.4	9.3	1.6	56.3
Treated with theelin	13.7	36.3	4.6	2.0	57.1

castration we observed the known effect of gonadectomy consisting in the increase of basophil cells and the appearance of a small percentage of castration cells (table 4). Under treatment with stilbestrol as well as estrone the formation of castration cells was not completely prevented but their number was diminished. The number of acidophil cells was decreased to approximately normal levels while the number of basophil cells remained higher in the castrated male animals. The gonadotropic potency of the pituitaries was tested in castrated animals using the technic of Lauson (33). Acetone-dried glands were extracted and the extracts injected into immature mice. Castrated animals showed a definitely demonstrable gonadotropic effect (table 5). This was reduced in the animals treated with stilbestrol as well as estrone. The reducing effect was more pronounced in the females than in the males and was higher in those treated with estrone than in the stilbestrol group.

Experiment 4. In a previous experiment (34) we were able to demonstrate a

TABLE 5. THE EFFECT OF STILBESTROL ON THE GONADOTROPIC POTENCY OF THE PITUITARY

	Mean uterine weight	Mouse uterine units	Units per av. pituitary	Potency comparison
	mg.			
Normal females	6.3 5.1 6.0	>1	>1	—
Castrated females	24.0 15.0 7.5	50	12.5	Increased 12.5×
Castrated females treated with stilbestrol	11.1 8.0 6.5	6	1.2	Reduced 10-fold
Castrated females treated with theelin	6.5 6.5 5.2	>1	>1	Reduced to normal
Castrated males	29.0 19.0 9.2	60	15	Increased 15-fold
Castrated males treated with stilbestrol	16.6 12.5 7.2	30	5	Reduced 3-fold
Castrated males treated with theelin	11.0 10.5 6.0	15	2.5	Reduced 7-fold

TABLE 6. THE EFFECT OF ESTRONE AND STILBESTROL IN RESISTANCE TO INFECTION

Hormone injected 7 days before infection	Average period (hours) of survival and number ( ) of survivors		
	1 mg. dose	0.1 mg. dose	0.01 mg. dose
Theelin, Parke, Davis	75.2 (2)	69.0 (1)	61.0 (0)
Stilbestrol, Winthrop	44 (0)	43.5 (0)	40.3 (0)
Control mice, sesame oil	32 (0)	31 (0)	31 (0)

definitely protective effect of estrone against the pneumococcus infection of mice. Using doses of 1 mg., 0.1 mg. and 1.01 mg. given 7 days before infection, we obtained a marked prolongation of the survival period of animals with the 1 mg. dose, 2 animals of this group surviving the infection. The same amount of stilbestrol did not show any protecting effect and no difference between the 3 doses was observed (table 6). No animal treated with stilbestrol survived the pneumococcus infection.

B. Experiments on the Toxicity of Stilbestrol in Animals

The lethal dose of diethylstilbestrol for various animals is independent of their relative size. Mice died rapidly after an intravenous dose of 175 mg. per kg. body weight (35). Rabbits succumbed quickly after a dose of 40 mg. per kg. Cats died overnight after a dose of 30 mg. per kg. Intraperitoneal injections of 500 mg. per kg. into mice led to death within 24 hours while doses of 200 mg. were well tolerated. Since the average clinical dose for the human being weighing at least 60 kg. is only 1 mg., the relative safety of its use seems well illustrated (Barlow). Chronic toxicity studies were reported by Loesser (36). A dose of 1 mg. per day produced in 44 to 54 days necrosis and fatty changes in the liver. In the kidneys of the rats similar changes were found as in severe jaundice. The authors also observed bleeding from the gut and the vagina and hemorrhage in the adrenal cortex. The appetite and motility of animals were slowed down, the skin appeared brown-red and pale, and they bled from the conjunctivae. The oxygen consumption of the animals decreased for the first 9 days and then returned to normal. The thyroid showed the picture of the resting gland. The cells of the adrenal glands contained no lipoid. Arnold (37) produced a marked decrease in reticulocytes and thrombocytes with an increase of leukocytes after daily intramuscular injections of 4 to 10 mg. in female and male dogs. At a certain level of the thrombocytopenia hemorrhages occurred followed by secondary anemia. After 30 to 40 days of continued therapy a condition of panmyelophthisis was reached. Tislowitz (38) too observed a disturbance of the myelogenic system with granulocytopenia and anemia in dogs receiving 5 mg. by injection for from 25 to 50 days. Selye (39) produced areas of necrosis in the liver of mice with doses of 1 mg. per day. Histological examination of his animals showed pneumonia, stomach ulcers, adrenal hyperplasia and pyometra. Only a small group of authors seem to emphasize the lack of severe toxicity to experimental animals. Teague (40) did not produce any liver necrosis in rats by giving doses of 0.1 gm. per kg. a day for 10 days. Grab emphasizes that more than a thousand times the effective dose is still harmless. Liver damage in rats was not observed in doses of 50

TABLE 7. EFFECT OF STILBESTROL UPON THE BODY WEIGHT OF CASTRATED ANIMALS

Type of animal	Average weight	Avg. wt. 1 mo. after	Gain (+) Loss (−)	Remark
	gm.	gm.	%	
Castrate females	102	139	+32	All animals gained weight
Treated, stilbestrol	197	182	−7.6	All but one animal lost weight
Treated, theelin	177	202	+14	60% of the animals gained weight
Castrate males	266	276	+3.7	All animals gained weight
Treated, stilbestrol	319	272	−15	All animals lost weight
Treated, theelin	309	299	−3.2	All but 2 animals lost weight

mg. per kg. (250,000 times the effective dose). Kreitmair and Sieckman produced no symptoms in a fox terrier with 300 mg. of the drug. These investigations emphasize that the importance of the toxicity of stilbestrol can be minimized since it is only observed in excessive doses. The question of the carcinogenic activity of this synthetic estrogen has been studied by Lacassagne (41), who used a mouse strain in which weekly injection of 50 gamma of

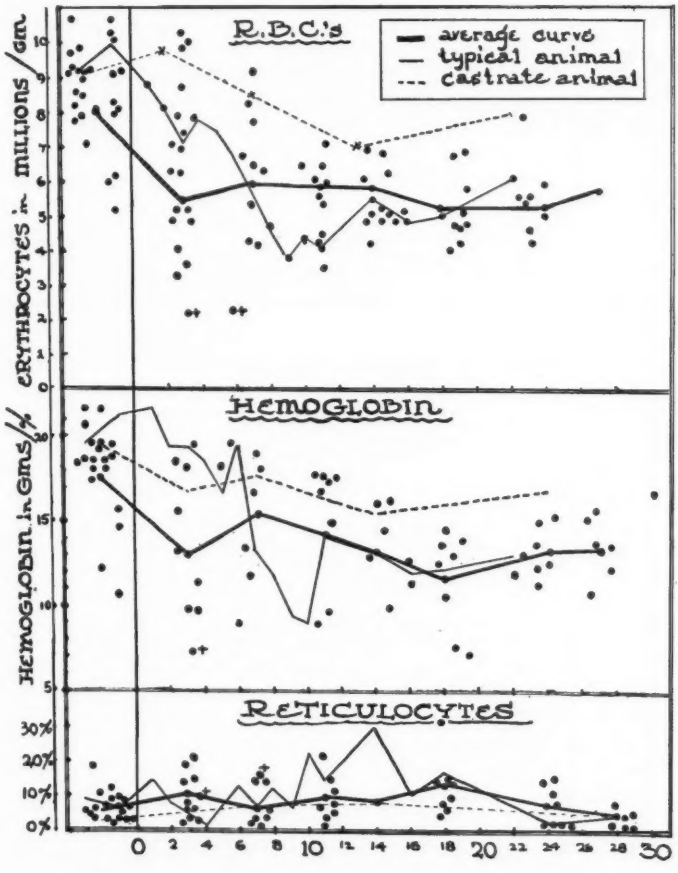

Fig. 2. SCATTERGRAM OF THE ERYTHROCYTE, THE HEMOGLOBIN AND THE RETICULOCYTES FOLLOWING THE ADMINISTRATION OF STILBESTROL.

estrone produced adenocarcinoma of the breast in the majority of males between 4 and 8 months. Two male mice injected with stilbestrol developed cancer of the breast 84 and 112 days after treatment, much earlier than they appeared in the estrone series.

From the foregoing brief review we can assume that definite toxic lesions have been produced with certain doses of this drug, although such doses were admittedly excessively high. Of the toxic changes the injury to the parenchymatous organs (liver and kidneys) and to the bone marrow seemed the most outstanding ones. Certain species of laboratory animals (dogs and rabbits) appeared more sensitive to stil-

bestrol than others, although the discrepancies in the doses used makes a comparison extremely difficult.

In our experiments comparative studies between the injurious effects of stilbestrol and estrone at doses commonly used in human therapy were made. Adult rats received 1 mg. and 0.1 mg. of either estrogenic substance 3 times a week for one month. Repeated, and in some instances daily, blood counts were made, and after a careful

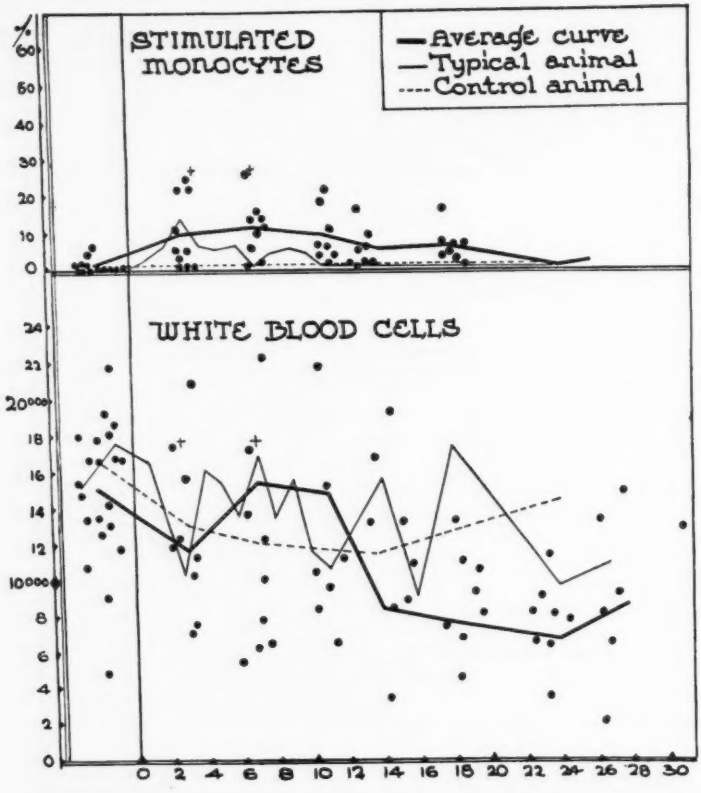

Fig. 3. Scattergram of the leukocytes following the administration of stilbestrol.

autopsy of the animals, which were killed after one month, the various organs were studied histologically.

Only 2 of our animals receiving the 1 mg. doses died spontaneously; all others, however, showed definite signs of impaired health. The fur was roughened and brown; hemorrhages of the conjunctivae were observed; the animals had bloody diarrhea or vaginal bleeding. They seemed nervous and excited and later lethargic. Animals receiving 0.1 mg. per dose showed no visible effects of toxicity except a loss of weight (table 7). The changes in the blood picture are recorded in the 3 scattergrams (fig. 2–4). A drop in red blood cells occurred in most animals receiving 1 mg., some of the counts dropping as low as 2,500,000. This drop in the red blood cells occurred during the first 2 weeks of experimentation and 8 of the 12 animals injected

with this dose showed later a slow rise of the number of erythrocytes to about 4,500,000 to 5,500,000, at which level they remained constant. Two animals died with severe anemia. The hemoglobin content of the blood similarly showed a noticeable drop about 5 to 7 days after initiation of the therapy. The reticulocyte response to this anemia was very marked, and in some animals a reticulocyte count up to 30% was obtained. The rise in reticulocytes usually occurred during the second and third weeks of the experiment and may be taken as proof that the regenerative power of the bone marrow is not impaired. The white blood cell counts in our experiments did not show any noticeable changes. In no instance could we find a significant leukopenia. In the differential counts of the white blood cells the relative rise of monocytes during the first week of treatment was interesting. The monocytes appeared in

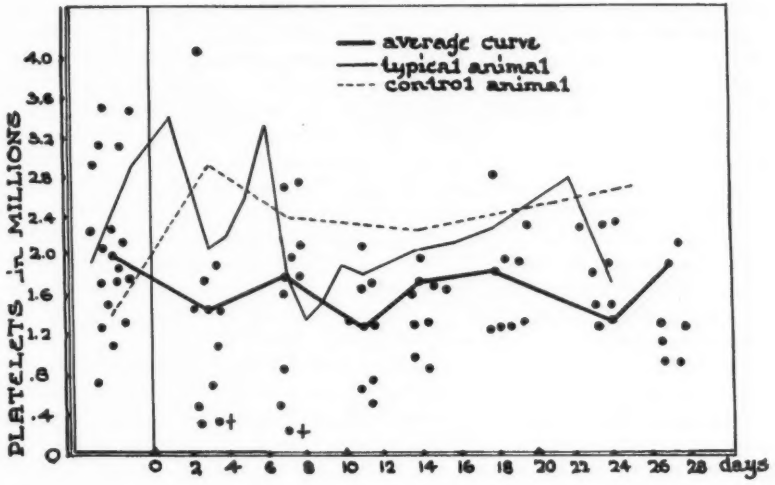

Fig. 4. Scattergram of the blood platelets following the administration of stilbestrol.

stimulated form (Sabin), showing large vacuoles with the vital staining process as proof of phagocytotic activity. This rise in stimulated monocytes was most marked in the animals which died. It preceded by about one week the rise of reticulocytes. The number of blood platelets was also at least briefly influenced by the stilbestrol treatment. Quite a number of individual counts during the first two weeks showed abnormally low values, as evident from the scattergram. In the two animals which died the thrombocytes fell below 150,000.

The histological examination of the bone marrow showed in all instances a very cellular marrow displaying hyperplasia rather than depression of hematopoiesis. In none of our animals did we obtain pictures of panmyelophthisis such as observed by Arnold or MacBryde. We must therefore assume that the first injury to the hemopoietic system occurs without too excessive doses of the drug in the peripheral blood, producing a certain degree of destruction of the red blood cells followed by the usual regenerative response from the bone marrow. At our dose level, and for the time of our observations, this peripheral damage of the blood cells could be checked sufficiently in all animals but 2 by increased bone marrow activity, although in no animal was the initial normal blood count achieved.

Histological examinations of our animals which died from the effects of the drug showed a picture similar to that presented by Loesser. There were necroses in the liver and severe degeneration of the tubular epithelium of the kidneys with heavy glomerular infiltration. The livers of the surviving animals showed a slight cellular anaplasia with some fatty degeneration in one instance. In the kidneys hyperemia of the glomeruli could be found with two cases of glomerular infiltration suggestive of an acute glomerular nephritis. The adrenals showed marked hyperplasia of the cortical zone with hyperemia which was so excessive that small blood lakes formed in the cortical zone. The adrenal cells appeared free of lipoid material, and in one of the animals which succumbed cortical necrosis was present. Aside from the marked proliferation of chromophobe cells in the pituitary, which we have discussed previously, marked hyperemia of this gland was noticeable in all our animals. In one case a focal necrosis in the anterior lobe was observed. Examination of the gastrointestinal tracts of the animals showed superficial necrosis of the mucous membrane of the small intestines in the animals which succumbed. The animals which had received the larger doses of 1 mg. but had survived occasionally showed an irregular patchy atrophy of the mucous membrane which in certain places was remarkably thinned. In 3 cases small ulcerations were observed grossly, which, however, on microscopic examination proved to be just circumscribed areas of mucosal atrophy. The colon and small intestine did not show any pathological changes. The thyroids of the animals appeared to be either normal or in a slight stage of colloidal involution. No changes were found in the heart muscle and nervous system of our animals. The lymph glands of the mesentery were moderately enlarged in some of our animals, and the microscopic examination showed hyperemia and an increase in the amount of hemosiderin pigment in the sinus endothelium of the lymph nodes. In the spleen an occasional increase of iron pigment was observed.

In conclusion we may then say that some evidence of toxicity in our animals was already present at doses as low as 0.1 mg. 3 times a week. This evidence consisted principally in a slight loss of weight and a blood count which was below the one found in the control rats. At doses of 1 mg. 3 times a week 2 fatalities occurred. They showed focal necrosis of liver, adrenal, intestines and pituitary. The other animals showed no changes suggesting any severe toxicity. The blood count in all animals was below the normal but remained constant without any further drop. In the kidney an occasional case of glomerulonephritis was observed. In the liver slight forms of cellular anaplasia were present. A slight increase of pigment in the spleen as well as in the lymph nodes may be taken as evidence of an increased blood destruction. In the bone marrow no depressing effect of the drug was observed. It would seem that with the exception of these 2 fatalities the changes found at our dose levels were by far not as drastic and alarming as those found by Loesser, who used only about twice the doses.

SUMMARY

The effects of equal doses of stilbestrol and theelin were studied in immature, mature and castrated animals.

The injection of stilbestrol in doses from 0.02 to 10γ produced an approximately equal effect on the immature mouse uterus as similar doses of theelin. At doses of 40γ and higher a marked discrepancy in the effect of both estrogenic substances was noted.

Stilbestrol produced a much more pronounced increase in the weight of the pituitary and adrenal in normal and castrated animals than equal doses of theelin.

Both substances led to a decrease in the number of acidophil and basophil cells

of the pituitary, which was more pronounced in castrated animals. The gonadotropic potency of the glands of castrated animals was more markedly decreased in the animals treated with theelin than in those treated with stilbestrol.

Stilbestrol did not exert any protecting influence on mice against pneumococcus infection such as has been demonstrated for similar doses of theelin.

A drop of red blood cells and hemoglobin occurred in most rats receiving 1 mg. of stilbestrol 3 times a week. This was followed in the surviving animals by an output of reticulocytes and stabilization of the red blood cells at a slightly lower level than normal. The blood platelets also showed a brief drop during the first weeks of treatment. The bone marrow did not show any depression of hematopoiesis.

In 2 animals which died during treatment severe changes in the liver and kidney as have been previously described were found. In the surviving animals slight parenchymatous degeneration of the liver, an occasional case of glomerulonephritis, and a patchy atrophy of the mucous membrane of the stomach were the most outstanding findings. They were already present at dose levels as low as 0.1 mg. 3 times a week.

We wish to express our deep gratitude to the Winthrop Chemical Company, the George A. Breon & Company and the Endo Products Company for furnishing the stilbestrol used in our investigation

REFERENCES

1. Dodds, E. C., and W. Lawson: *Nature* 139: 627. 1937.
2. Preliminary report of the Council on Pharmacy and Chemistry: *J.A.M.A.* 113: 2312. 1939.
3. Shorr, E., F. H. Robinson and G. N. Papanicolaou: *J.A.M.A.* 113: 2312. 1939.
4. Buxton, C. L., and E. T. Engle: *J.A.M.A.* 113: 2318. 1939.
5. MacBryde, C. M., H. Freedman and E. Loeffel: *J.A.M.A.* 113: 2320. 1939.
6. Dodds, E. C., W. Lawson and R. L. Noble: *Lancet* 234 (1): 1389. 1938.
7. Kreitmair, H., and W. Sieckman: *Klin. Wchnschr.* 18: 156. 1939.
8. Emmens, C. W.: *J. Physiol.* 94: 22P. 1939.
9. Leighty, J. A., and H. J. Wick: *Endocrinology* 25: 597. 1939.
10. Varangot, J.: *Presse med.* 47: 725. 1939.
11. Sondern, E.: Personal communication.
12. Grab, W.: *München. med. Wchnschr.* 86: 436. 1939.
13. Palmer, A.: *Lancet* 236 (1): 933. 1939.
14. Gros, G.: *Compt. rend. Soc. de Biol.* 131: 172. 1939.
15. Parkes, A. S., E. C. Dodds and R. L. Noble: *Brit. M. J.* 2: 557. 1938.
16. Düffner, G. J., and R. W. Whitehead: *Scient. Proc. Am. Soc. for Pharmacol. & Exper. Therap.*, 31st Ann. Meet., New Orleans, La., March 13–16. 1940.
17. Folley, S. J., and H. M. S. Watson: *Lancet* 235 (2): 423. 1938.
18. Raynaud, A.: *Compt. rend. Soc. de Biol.* 131: 218. 1939.
19. Wolff, M. E.: *Compt. rend. Acad. de Sc.* 208: 1532. 1939.
20. Noble, R. L.: *J. Physiol.* 94: 177. 1938.
21. Noble, R. L.: *Lancet* 235 (2): 192. 1938.
22. Jacobsen, E.: *Endokrinol.* 21: 20. 1938.
23. Mellish, C. H., A. J. Baer and A. C. Macias: *Endocrinology* 26: 693. 1940.
24. Sherwood, T. C.: *Endocrinology* 26: 693. 1940.
25. Cobet, R., M. Ratschow and M. L. Steckner: *Klin. Wchnschr.* 18: 278. 1939.
26. Dawson, R. F., and J. M. Robson: *J. Physiol.* 95: 420. 1939.
27. Hoskins, R. G., and R. Small: Advance Abstract, *Endocrinology* 26: 730. 1940.
28. Zondek, B., and L. Marx: *Nature* 143: 378. 1939.
29. Mühlbock, O.: *Nature* 143: 160. 1939.
30. Duyvene de Wit, J. J., and L. H. Bretschneider: *Klin. Wchnschr.* 18: 1423. 1939.
31. Fluhmann, C. F.: *Am. J. Physiol.* 95: 422. 1930.
32. Wolfe, J. M.: *Am. J. Physiol.* 115: 665. 1936.
33. Lauson, H. D., J. B. Golden and E. L. Severinghaus: *Endocrinology* 25: 1. 1939.
34. von Haam, E., and I. Rosenfeld: Unpublished data
35. Barlow, O. W.: Unpublished data.
36. Loesser, A.: *Ztschr. f. d. ges. exper. Med.* 105: 430. 1939.
37. Arnold, O.: *Klin. Wchnschr.* 18: 891. 1939.
38. Tislowitz, R.: *Acta brev. Neerland.* 9:15. 1939.
39. Selye, H.: *Canad. M. A. J.* 41: 48. 1939.
40. Teague, R. S.: *Scient. Proc. Am. Soc. Pharmac. & Exper. Therap.*, 31st Ann. Meet., New Orleans, La., March 13–16. 1940.
41. Lacassagne, A.: *Compt. rend. Soc. de Biol.* 129. 641. 1938.

A QUANTITATIVE STUDY OF THE INHIBITION OF ESTRADIOL BENZOATE BY PROGESTERONE IN THE BABOON (*PAPIO PORCARIUS*)

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THE QUANTITATIVE INHIBITION of estrogen by progesterone has been estimated exclusively by means of histological examination of the endometrium in primates. In the baboon, however, it is possible to determine the quantitive inhibition of the two ovarian hormones by means of the sexual skin and the time of the onset of bleeding after the last injection of the combined hormones.

When estradiol benzoate is administered to a prepubertal or castrate baboon the perineum swells or turgesces, and with effective doses it reaches its maximum size in 7 to 10 days. When the hormone is withheld the perineum returns to its resting level about the 10th day, and between the 10th to 16th days after withdrawal of the estrogen bleeding supervenes (2, 3).

When it was discovered that progesterone, given to a mature baboon in the first part of the cycle, causes the turgescent perineum to undergo deturgescence (4, 5) a new, non-operational technic became available for assessing by simple external measurement the quantitative inhibition of estrogens by progesterone in the baboon.

In a preliminary communication (6) we demonstrated that if a prepubertal baboon, previously treated with 1 mg. estradiol daily for 10 days, was injected daily with combinations of 1 mg. estradiol and varying quantities of progesterone for 10 days, one of two reactions might be evoked. If adequate quantities of progesterone (2 mg. daily) were employed then the perineum returned to a state of rest by the 10th day and bleeding occurred 2 to 5 days after the suspension of both injections. The other response manifested itself when inadequate quantities of progesterone (1 mg. daily) were administered; the perineum did not as a rule reach the resting level and bleeding only supervened 10 to 16 days after withdrawal of both hormones.

These experiments indicated to us that the state of the perineum and the time of onset of bleeding could be used as reliable indicators of effective inhibition of estradiol by progesterone. It is possible however that the sexual skin may not parallel the changes in the endometrium. The relation between the uterus and the sexual skin under these experimental conditions will be established later, but, whether there is any correlation or not between the responses in these two tissues, there is no doubt that the state of the sexual skin and the time of onset of bleeding can be used in quantitative experiments involving the use of estradiol and progesterone.

We discovered subsequent to our preliminary note that 2 mg. of progesterone could inhibit not only 1 mg. but as much as 5 mg. and even 10 mg. of estradiol benzoate. These experiments proved that progesterone does not inhibit estradiol in a

Received for publication August 20, 1940.

quantitative ratio; but that once an optimal concentration of progesterone is attained, it will inhibit extraordinarily large quantities of estradiol benzoate.

The continuation of these experiments is the object of record in this paper.

MATERIAL AND METHODS

In this investigation a total of 43 experiments were performed on 25 baboons, of which 18 were immature and 7 were adults. Twenty-five experiments were conducted on the immature, and 18 experiments on the mature animals.

The mature animals include 2 young lactating baboons and a young pubescent animal that had been used some months previously while it was still immature. All these adult female baboons including the pubescent animal were castrated. In one lactating baboon experiments were performed both before and after castration (cases 41–44, table 6).

From tables 1 to 6 it will be observed that a total of 49 cases are quoted whereas only 43 experiments were performed. This discrepancy is due to the fact that in 6 instances a single experiment served to prove 2 different points. Thus for example *case 12*, table 2, is the same experiment as *case 26*, table 5.

The hormone used in these investigations is manufactured by Schering under the trade name of progynon-B oleosum (estradiol benzoate) and proluton (progesterone). One mg. of progynon is equivalent to 10,000 international benzoate units, and 1 mg. of proluton is equivalent to 1 international unit of progesterone.

Unless otherwise stated all animals were injected daily with 1 mg. estradiol benzoate for 10 days in order to produce a maximal perineal turgescence. On the 11th day the injections of estradiol benzoate combined with progesterone were commenced and they were given daily for a further 10 days. In some animals for reasons mentioned below the combined injections were continued for 16 or 20 days.

The perineum of each animal was measured daily throughout the course of the experiment. Vaginal smears and uterine biopsies were also made, but these will be fully described in a separate paper.

The criteria used to ascertain the effectiveness of progesterone are 2-fold (1) the state of the perineal swelling and (2) the period intervening between the withdrawal of the combined hormones and the appearance of external macroscopic bleeding.

When progesterone is effective the perineum returns to a state of rest by the 10th day after the administration of the combined estradiol benzoate and progesterone and bleeding occurs within 2 to 5 days after the last injection. This type of bleeding we have called provisionally 'progesterone withdrawal bleeding.' Per contra when progesterone is ineffective the perineum does not as a rule reach the resting level and bleeding only supervenes 10 to 16 days after withdrawal of both hormones. This type of bleeding we have called provisionally 'estrogen-like withdrawal bleeding,' because the time of onset of bleeding is identical with true estrogen withdrawal bleeding produced in an animal injected only with estrogen.

The perineum is a very sensitive indicator, and in the majority of experiments we were able to predict by observing the perineum whether the progesterone would be effective or not. In 3 cases (*12*, *38*, *46*, however, perineal rest had been achieved but the bleeding set in after a latent period, which showed that progesterone was insufficient to overwhelm completely the estradiol.

EXPERIMENTAL

First experiment (table 1, fig. 1). This experiment was planned to determine firstly, the effect of estradiol benzoate alone on the perineum of immature baboons

and secondly, to ascertain the time of onset of bleeding after the withdrawal of estradiol. The amount of estradiol injected daily, namely, 1 mg., was well above the threshold level for the baboon.

The animals were given 1 mg. of estradiol benzoate daily for varying periods.

The results of this experiment are summarized in table 1. The perineum commences to swell on the third day, and reaches its maximum on the 10th day. If the estradiol is continued the perineum remains turgescent (fig. 1, A). When the estrogen

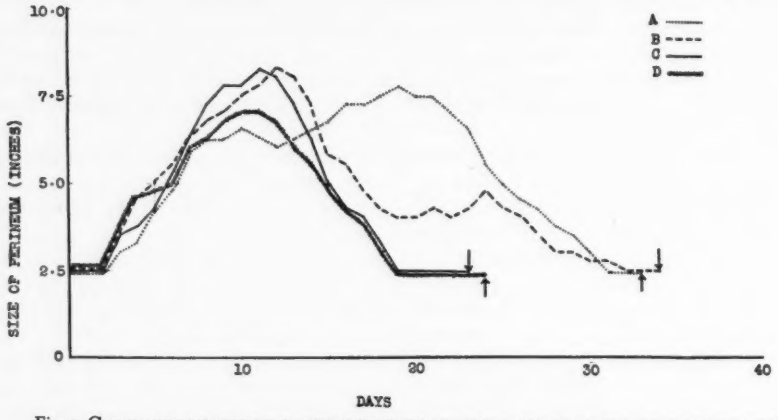

Fig. 1. Curves showing effects of estradiol benzoate alone and of combinations of estradiol benzoate and progesterone on the perinum of the immature baboon.

A, 1 mg. estradiol benzoate only given for 20 days (*case 5*)

B, 1 mg. estradiol benzoate daily for 10 days, followed by combined injections of 1 mg. estradiol benzoate and 1 mg. progesterone daily for a further 10 days (*case 13*)

C, 1 mg. estradiol benzoate daily for 10 days, followed by combined injections of 1 mg. estradiol benzoate and 2 mg. progesterone daily for a further 10 days (*case 10*)

D, Similar to C except that 5 mg. estradiol benzoate was substituted for 1 mg. in the combined injections (*case 18*)

The arrows in each case represent the onset of external macroscopic bleeding.

is withdrawn the perineum remains stationary for 1 to 3 days, but it returns to its resting level 10 to 12 days later; external bleeding invariably follows 10 to 16 days after the last injection of estradiol. This is typical of estrogen withdrawal bleeding in the baboon.

Second experiment (table 2, fig. 1). When an adequate quantity of progesterone is administered to mature baboons in the first part of the cycle it will cause the turgescent perineum to undergo deturgescence (4, 5). *Experiment 1* proved that the perineum of an immature animal may be made to turgesce by means of estradiol ben-

Table 1. The effect of estradiol benzoate alone in immature baboons

Case No.	Estradiol benzoate only	State of perineum on cessation of injections	Time of bleeding after last injection
	mg.		*days*
1	1 (10 days)	Turgescent	10
2	1 (10 days)	Turgescent	16
3	1 (10 days)	Turgescent	12
4	1 (16 days)	Turgescent	12
5	1 (20 days)	Turgescent	13
6	1 (20 days)	Turgescent	15

zoate and, provided the estradiol benzoate is continued, the turgescence may be maintained indefinitely (fig. 1, A).

The next phase in this investigation was to determine precisely the daily amount of progesterone required to antagonize effectively 1 mg. of estradiol in immature baboons that had previously been treated for 10 days with 1 mg. estradiol benzoate per day.

Progesterone given in 3 mg. (*case 7*) or 2 mg. (cases 8–11) quantities combined with 1 mg. of estradiol daily led to a steady but continuous decrease in size of the turgescent perineum until the 8th to 10th days when a state of rest was reached; bleeding set in 3 to 4 days after withdrawal of the combined injections (fig. 1, C). With 1.5 mg. progesterone (*case 12*) the perineal deturgescence was complete in 10 days, but bleeding occurred 10 days after withdrawal. Where only 1 mg. of proges-

TABLE 2. THE EFFECT OF COMBINED INJECTIONS OF FIXED QUANTITIES OF ESTRADIOL BENZOATE WITH VARYING QUANTITIES OF PROGESTERONE IN THE IMMATURE BABOON, PREVIOUSLY TREATED WITH 1 MG. OF ESTRADIOL BENZOATE DAILY FOR 10 DAYS

Case No.	Combined estradiol benzoate and progesterone[1]		Perineal deturgescence on cessation of injections	Time of bleeding after last injection
	Estradiol benzoate	Progesterone		
	mg.	*mg.*		*days*
7	1	3	Complete	4
8	1	2	Complete	4
9	1	2	Complete	4
10	1	2	Complete	3
11	1	2	Complete	3
12	1	1.5	Complete	10
13	1	1	Incomplete	15
14	1	1	Incomplete	16
15	1	1	Incomplete	12
16	1	1	Incomplete	13

[1] Combined estradiol benzoate and progesterone administered daily for 10 days to animals previously treated with 1 mg. estradiol benzoate daily for 10 days.

terone was used (cases 13–16) perineal deturgescence was incomplete by the 10th day of the combined injections, and bleeding occurred 10 to 16 days after withdrawal (fig. 15, B).

Thus 3 mg. or 2 mg. progesterone combined with 1 mg. of estradiol benzoate causes a complete perineal deturgescence and a bleeding which occurs within 3 to 4 days after withdrawal, i.e. progesterone withdrawal bleeding; whereas 1 mg. progesterone leads to incomplete perineal deturgescence accompanied by a bleeding which occurs 10 to 16 days after withdrawal, i.e. estrogen-like withdrawal bleeding (fig. 1, B & C).

Experiment 3 (table 3). The question now arose as to whether there exists a quantitative ratio between the amount of progesterone and estradiol benzoate necessary to cause that complete perineal deturgescence and progesterone withdrawal bleeding. That is, if the estradiol were increased, would a proportionate increase of progesterone be essential to produce the two effects described above; and conversely, if the estradiol benzoate were diminished, was a proportionate diminution of the progesterone required?

The results of this experiment are shown in table 3. Despite the fact that the estradiol benzoate had been increased by as much as 5 (cases 18 and 19) and 10 times (*case 17*), 2 mg. of progesterone was capable of producing complete perineal deturgescence and progesterone withdrawal bleeding in the same way as it did when com-

TABLE 3

Case No.	Combined estradiol benzoate and progesterone[1]		Perineal deturgescence on cessation of injections	Time of bleeding after last injection
	Estradiol benzoate	Progesterone		
	mg.	*mg.*		*days*
17	10	2	Complete	4
18	5	2	Complete	4
19	5 (16 days)	2 (16 days)	Complete	5
20	0.5	1	Incomplete	15

[1] Combined estradiol benzoate and progesterone administered daily for 10 days (except in *case 19*) to animals previously treated with 1 mg. estradiol benzoate daily for 10 days.

bined with only 1 mg. of estradiol (fig. 1, D and C). When the estradiol and the progesterone however, were proportionately diminished to 0.5 mg. and 1 mg. respectively (*case 20*) then the perineal deturgescence was incomplete and bleeding set in 15 days after withdrawal of the combined injections.

The only conclusion we could draw from this experiment was that there was no quantitative relationship between the relative amounts of estradiol and progesterone required (when combined) to produce perineal deturgescence and progesterone withdrawal bleeding. In fact we are of the opinion from these experiments that once the optimal quantity of progesterone is given it can counteract relatively enormous amounts of estradiol. The greatest absolute quantity of estradiol benzoate that can be antagonized by 2 mg. progesterone has not been determined on account of the prohibitive cost of this drug; the least amount, also, could not be accurately defined because, once the amount of estradiol approximates the threshold level for the baboon, a new problem arises which lies outside the scope of this paper.

Experiment 4 (table 4). Hitherto the combined injections of estradiol and progesterone had been continued for 10 days only, and then the question naturally arose as to whether or not optimum quantities of progesterone completely suppressed the action of estradiol and consequently the bleeding (which later supervened after the suspension of both hormones) was in fact of the estrogen withdrawal type seen in table 1. More especially did this seem possible as the bleeding occurred 13 to 15 days after the initiation of the combined injections.

Accordingly the following experiment was performed. After the baboons had received 1 mg. estradiol benzoate daily for 10 days, various combinations of estradiol benzoate and progesterone were given for 16 and 20 days instead of the customary 10 days (table 4).

The results of this experiment proved that, provided the optimum quantity of

TABLE 4

Case No.	Combined estradiol benzoate and progesterone[1]		Perineal deturgescence on cessation of injections	Time of bleeding after last injection
	Estradiol benzoate	Progesterone		
	mg.	*mg.*		*days*
21	1 (16 days)	2 (16 days)	Complete	3
22	5 (16 days)	2 (16 days)	Complete	5
23	1 (20 days)	2 (20 days)	Complete	3
24	1 (16 days)	1 (16 days)	Incomplete	16

[1] Combined estradiol benzoate and progesterone administered daily for 16 and 20 days to animals previously treated with 1 mg. estradiol benzoate daily for 10 days.

progesterone were given (cases 21–23), it did not matter how much longer the injection period extended beyond the customary 10 days, because progesterone withdrawal bleeding invariably occurred, whereas if insufficient progesterone were administered for the same period, then estrogen-like withdrawal bleeding would set in (*case 24*).

Experiment 5 (table 5). In all the experiments where the optimum progesterone effect was achieved 2 mg. of progesterone were given for 10 days; and it seemed that perhaps the total quantity of progesterone might be the factor that was responsible for producing this optimum action. The results of the experiment to prove this point are summarized in table 5.

If the total dose of progesterone was 20 mg. (cases 28, 29) then progesterone withdrawal bleeding occurred. Any quantity less than 20 mg. progesterone might cause

TABLE 5

Case No.	Combined estradiol benzoate and progesterone[1]				Perineal deturgescence on cessation of injections	Time of bleeding after last injection
	Estradiol benzoate	Total	Progesterone	Total		
	mg.	mg.	mg.	mg.		days
25	1 (10 days)	10	1 (10 days)	10	Incomplete	16
26	1 (10 days)	10	1.5 (10 days)	15	Complete	10
27	1 (16 days)	16	1 (16 days)	16	Incomplete	16
28	1 (20 days)	20	1 (20 days)	20	Complete	3
29	1 (10 days)	20	2 (10 days)	20	Complete	4

[1] Combined estradiol benzoate and progesterone administered to animals previously treated with 1 mg. estradiol benzoate daily for 10 days.

complete deturgescence (*case 26*) or incomplete deturgescence (*cases 25, 27*) of the perineum, but the bleeding was always of the estrogen withdrawal type.

Castrate Baboons

Hitherto all the experiments had been performed on immature animals with intact ovaries. In order to satisfy ourselves that the ovaries in prepubertal baboons were non-functional we decided to castrate adult female baboons (table 6).

After 10 days treatment with 1 mg. estradiol benzoate followed by a continuation of 1 mg. estradiol and 2 mg. progesterone for 10 days, the perineum underwent complete deturgescence and progesterone withdrawal bleeding occurred in 2 animals (cases 33 and 40). *Case 33* was a very young animal that had been used in previous experiments on immature baboons (cases 30, 31); it later went into puberty and after several normal menstrual cycles was castrated. *Case 40* was a young lactating baboon.

However, in three animals (cases 36, 41 and 45) incomplete deturgescence of the perineum and estrogen-like withdrawal bleeding supervened. When the amount of progesterone was increased to 3 mg. the estradiol was completely antagonized in 2 animals (cases 37, 42) while in the third animal (cf. cases 45–47) as much as 4 mg. of progesterone were required to produce the optimum progesterone effect. Two other animals (cases 48 and 49) showed that 4 mg. and 3 mg. of progesterone, respectively were effective in antagonizing 1 mg. of estradiol.

Having determined the optimum amount of progesterone required by each individual castrate animal to antagonize 1 mg. estradiol we then increased the amount of estradiol benzoate (cases 34, 35, 39, 44) as had been done in the immature animals (table 3). Except for *case 39*, the increased amount of estradiol benzoate was antag-

onized by the same amount of progesterone as had been required to inactivate optimally 1 mg. estradiol benzoate by each animal.

The exceptional case also reacted inconsistently here since 3 mg. was originally effective (*case* 36) while later 4 mg. progesterone proved only partially effective (*case* 37).

One feature worth mentioning is that young lactating uncastrated baboons respond to estradiol benzoate and progesterone in the same way as castrated baboons (cases 40, 41, 42). One lactating baboon was castrated and it responded in the same way to estradiol benzoate as it had done prior to castration (cf. cases 42, 43).

With the exception of one animal (cases 36–39), to which reference has already

TABLE 6. THE EFFECT OF COMBINED DAILY INJECTIONS OF ESTRADIOL BENZOATE AND PROGESTERONE GIVEN FOR 10 DAYS TO 7 ADULT CASTRATE BABOONS PREVIOUSLY TREATED WITH 1 MG. ESTRADIOL BENZOATE DAILY FOR 10 DAYS. EXPERIMENTS PERFORMED ON THE SAME ANIMAL ARE BRACKETED. (P)=IMMATURE. (L)=LACTATING.

Case No.	Combined estradiol benzoate and progesterone		Perineal deturgescence on cessation of injections	Time of bleeding after last injection
	Estradiol benzoate	Progesterone		
	mg.	*mg.*		*days*
30 (P)	1	1	Incomplete	16
31 (P)	1	2	Complete	4
32	1	1	Incomplete	10
33	1	2	Complete	3
34	5	2	Complete	5
35	5	2	Complete	5
36	1	2	Incomplete	10
37	1	3	Complete	5
38	1	4	Complete	7
39	5	3	Incomplete	11
40 (L)	1	2	Complete	3
41 (L)	1	2	Incomplete	8
42 (L)	1	3	Complete	3
43	1	3	Complete	4
44	5	3	Complete	4
45	1	2	Incomplete	16
46	1	3	Complete	16
47	1	4	Complete	5
48	1	4	Complete	4
49	1	3	Complete	4

been made, all the castrated animals reacted to combinations of estradiol benzoate and progesterone in a manner essentially similar to that characteristic for the immature baboons, except that a greater quantity of progesterone was required to produce an optimum antagonism of estradiol. This increased resistance to progesterone shown by mature baboons is most likely related to the age of the animal. A young lactating baboon (*case* 40) and a pubescent animal (*case* 33) when castrated responded effectively to 2 mg. of progesterone. Older animals required corresponding larger quantities of progesterone to produce an optimum antagonism of estradiol, e.g. *case* 43 required 3 mg. and *case* 47 4 mg. of progesterone. *Case* 36, the oldest animal in the colony, reacted inconsistently and in one experiment (*case* 38) even 4 mg. of progesterone was only partially effective.

DISCUSSION

The quantitative antagonism of estradiol benzoate to progesterone. In a preliminary note we discussed briefly the quantitative inhibition of estradiol benzoate by pro-

gesterone in the baboon as compared with other animals (6). For the comparative table submitted there we collated the results of other investigators who had used neither the same standardized hormones, nor the same species of animal. The results were compared by reducing them to a common denominator. Robson (9, 10) performed quantitative experiments on the mouse and the rabbit; Leonard, Hisaw and Fevold (8) used the rabbit and Hisaw (7) in addition experimented with these hormones on the monkey. In these cases the common denominator was the rabbit. We were thus able to express mathematically the sensitivity of each animal to progesterone as follows baboon:monkey:rabbit:mouse::7500:1000:50:1.

After our correlational observations wer published we obtained a paper by Courrier and Gros (1) who had performed quantitative experiments on the Algerian monkey, the rabbit and the rat. These investigators found that in order to antagonize a fixed quantity of estrogen the monkey required 6 times that amount of progesterone, the rabbit 40 and the rat 125 to 400. Thus the Algerian monkey proved $6\frac{1}{2}$ times more sensitive to progesterone than the rabbit, while the rat was 3 to 10 times less sensitive than the rabbit.

Incorporating these observations in our original scheme we thus obtain the following baboon:monkey:Algerian monkey:rabbit:rat:mouse::7500:1000:325:50:17–5:1.

At the time we published these comparative figures we were aware of the criticisms that might be raised in this connection. At the same time our preliminary experiments suggested that in the baboon estradiol benzoate was antagonized quantitatively by progesterone. However, in *experiment* 3 (table 3) it has been demonstrated that, in the baboon, there is no true quantitative antagonism in the sense that Robson affirms for the rabbit. Robson (10) states "In as far as can be judged from the results obtained the ratio of the two hormones given is the essential factor in determining what degree of luteal proliferation is produced when estrone plus progesterone (in adequate amounts) act on the uterine endometrium."

The impression obtained from Robson's statement is that, in the rabbit, the quantitative inhibition of estrogen by progesterone is a proportionate ratio, i.e. if A mg. of progesterone inhibited B mg. of estrogen then 2 A mg. of progesterone will inhibit 2 B mg. of estrogen.

In the baboon a daily dose of 2 mg. of progesterone will inhibit 1.0 to 10.0 mg. estradiol benzoate. The antagonism between estradiol benzoate and progesterone in the baboon is thus different from that recorded for the rabbit and other animals in that the baboon obeys the biological 'all or none' law rather than a strictly mathematical law of direct proportional variation.

Estrogen withdrawal bleeding and progesterone withdrawal bleeding. Estrogen withdrawal bleeding in the baboon occurs 10 to 16 days after the administration of the last injection of the estrogen. When adequate quantities of progesterone are given simultaneously with estrogen to an immature or a castrated baboon which has previously been treated with estrogen and when subsequently both hormones are withdrawn, then bleeding occurs, but in this instance it supervenes within 3 to 5 days. In *experiment* 2 (table 2) it has been demonstrated that 1 mg. of estradiol benzoate given simultaneously with 2 mg. progesterone over 10 days will cause progesterone withdrawal bleeding. On the other hand a daily dose of 1 mg. estradiol and 1 mg. (or 1.5 mg.) of progesterone given for 10 days produces bleeding of the estrogen withdrawal type, which we have called estrogen-like withdrawal bleeding. Even if 1 mg. progesterone is given for 16 days such estrogen-like withdrawal bleeding supervenes. But if 1 mg. progesterone is given for 20 days, then only is a progesterone withdrawal bleeding precipitated. This means that a total of 20 mg. of progesterone given

over 10 to 20 days is necessary to cause progesterone withdrawal bleeding. It may also be mentioned that in the mature baboon 15 mg. of progesterone in divided doses, or 20 mg. as a single dose given in the first part of the normal cycle will lead to premature bleeding 4 to 7 days after the last injection (4, 5). It seems therefore that in the castrate, immature and non-castrated mature baboons bleeding produced by the withdrawal of progesterone is dependent to a large extent on the total quantity of progesterone; the minimum quantity required for this purpose is 20 mg. In very old castrated animals the total quantity of progesterone rises to 30 to 40 mg. and while it is not possible to state with certainty, there is a likelihood that as the baboon ages it becomes less sensitive to progesterone.

SUMMARY

The quantitative antagonism of estradiol benzoate and progesterone has been examined in immature and adult castrate baboons. The criteria used for the successful inhibition of estradiol benzoate by progesterone are the complete deturgescence of the turgescent perineum caused by estradiol and the production of progesterone withdrawal bleeding when both hormones are suspended.

Two mg. of progesterone administered daily for 10 days with 1.0 to 10 mg. of estradiol benzoate to an immature baboon previously treated for 10 days with estradiol will cause complete perineal deturgescence and progesterone withdrawal bleeding.

In the castrated baboon the effective daily dose of progesterone necessary to antagonize 1 mg. of estradiol simultaneously administered varies from 2 to 4 mg. depending probably on the age of the animal.

There is no proportional relationship between the antagonism of estradiol benzoate and progesterone in the immature and adult castrate baboon such as occurs in other animals. Once there is an adequate quantity of progesterone available large quantities of estradiol benzoate (5 to 10 mg. daily) will be effectively antagonized.

The successful antagonism of estradiol benzoate is dependent on the total quantity progesterone used; in immature animals a minimum of 20 mg. is required as contrasted with 20–40 mg. for young and old adult castrate baboons respectively.

We wish to express our deep appreciation to Professor Raymond A. Dart for his advice and criticism and for the excellent facility provided in his department, and to Dr. G. Buchanan for his interest and encouragement.

Messrs. Scherag (Pty.) Ltd. have generously supplied in part the hormones used in the present study.

This investigation was aided by a grant made by the National Research Council and Board of the Union of South Africa to whom our thanks are due.

REFERENCES

1. Courrier and Gros: *Compt. rend. Soc. de Biol.* 125: 746. 1937.
2. Gillman, J.: *S. Afr. J. Med. Sci.* 2: 161. 1937.
3. Gillman, J.: *S. Afr. J. Med. Sci.* 3: 66. 1938.
4. Gillman, J.: *Endocrinology* 26: 80. 1940.
5. Gillman, J.: *Endocrinology* In Press.
6. Gillman, J., and H. B. Stein: *Nature* 143: 559. 1939.
7. Hisaw, F. L.: *Am. J. Obst. & Gynec.* 29: 638. 1936.
8. Leonard, S. L., F. L. Hisaw and H. L. Fevold: *Am. J. Physiol.* 100: 111. 1932.
9. Robson, J. M.: *J. Physiol.* 92: 371. 1938.
10. Robson, J. M.: *J. Physiol.* 88: 100. 1936.

INCREASED RESISTANCE TO SYPHILIS IN THE RABBIT FOLLOWING PROLONGED ADMINISTRATION OF URINARY ESTROGENS

I. FEMINIZING EFFECTS OF ESTROGENS ON ADULT MALE RABBITS

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THIS PAPER is concerned with a study bearing on the relationship between sex and immunity to syphilis. The origin of the problem lies primarily in a body of clinical observations which show that syphilis in many respects is a much milder disease in women than in men, and that pregnancy apparently plays an important role in activating, or in enhancing, the defensive reaction against this disease.

Our immediate interest in the problem arose through contact with a case of severe secondary syphilis in a multiparous Chinese woman who had passed through the menopause before contracting the disease. The failure of sex and of many pregnancies to afford protection against an infection incurred at this late period of life attracted our attention. There was to be considered the probable importance of the waning sexual functions as a factor influencing the state of susceptibility.

The classical observations of Colles (1) in 1837, that apparently normal women suckled with impunity their syphilitic offspring, may be said to have laid the foundation of the conception that pregnancy exercises a modifying influence on the course of syphilis. The fact that Colles was unaware of the presence of the disease among the women he observed only emphasizes the implications of the phenomenon. Hutchinson (2) in 1876 reached the conclusion that all mothers of syphilitic children were themselves syphilitic, and that pregnancy acted to suppress the reaction to infection in the female, and alter the rhythm of its course.

Many years later Brown and Pearce (3) suggested that pregnancy is a factor which influences the severity of the disease. They found that the pregnant rabbit inoculated at the time of conception reacted to infection less severely than the normal female animal, and that this difference extended through the period of pregnancy and well into the period of lactation. Similar findings were reported by Chesney (4) and confirmed later by Kemp (5).

Further reason for attributing to pregnancy a part in the defensive mechanism of the female is found in clinical reports (6–10). From a study of human cases Moore (6, 7, 8) found that pregnancy may cause either complete suppression of all early lesions of syphilis, or a disturbance in the time relationships of the successive stages of the disease. This protective influence was also manifest in the more frequent occurrence of latency, and in the lower incidence of neurosyphilis among women. Moore also came to the conclusion that the protection afforded by pregnancy often extends over a long period of time. Solomon (9) thought it probable that multiple pregnancies were more effective in this respect than a single pregnancy.

That pregnancy apparently enhances natural resistance to syphilis further emphasizes the importance of the sexual factor to the defensive reaction. We have assumed for the reasons stated above that some endocrine function peculiar to the female, and intimately related to gestation, plays a dominant role in the defensive mechanism. One of the striking phenomena of pregnancy is the elaboration of large quantities of estrogens.

Received for publication August 2, 1940.

These can be detected in the blood (11) and in the urine (12) in increasing amounts as pregnancy progresses. After parturition they fall rapidly to the low level of the intermenstrual period, and with the decline of sexual function at the menopause, they gradually disappear, or are reduced to a very low level.

With the possibility in mind that estrogenic hormones might have important functions in the development of the body's resistance to syphilis, either directly or indirectly, these experiments were undertaken. Adult male rabbits subjected to the action of urinary estrogens were infected with *Treponema pallidum*. In the first of two experiments, the early physiological effects of the estrogenic preparation were found to coincide with a modification of the severity of the disease (13). These results led us to the opinion that the influence of estrogens on infection was indirect and probably related to the tissue changes which they induced, especially those in the testes. Upon this assumption, a second experiment was performed in which inoculation was deferred until there was evidence of the full physiological effects of the estrogens. These included well defined signs of feminization as exhibited by changes in the sexual organs, and by the development of secondary sexual characteristics.

Inasmuch as the course of disease in the second experiment did not differ essentially from that of the first, the animals of both experiments have been combined for the purpose of analysis. When there were variations between the two experiments which were apparently related to the difference in the duration of treatment, the results were considered separately.

MATERIALS AND METHODS

Preparation of estrogenic substance. The estrogenic substance employed was prepared from the same lot of human pregnancy urine collected from Chinese women admitted to the obstetrical service (for the most part during the last third of pregnancy). Collection was continued over a period of 3 months. Each portion of urine was acidified to congo red on the day of collection by the addition of glacial acetic acid. After collection, the entire lot of urine stood at room temperature for a period of 4 months before the extraction of estrogenic substance was begun. No preservative was added. The acidified urine was extracted with butyl alcohol in a continuous extractor like that described by Veler, Thayer, and Doisy (14). During this procedure, the extract was heated continuously to a temperature of 100°C for at least 12 hours. Afterwards, it was stored in a glass flask in a refrigerator at 5°C, and at intervals of time portions were removed and prepared for assay, and for use in treatment. Whether for assay or treatment, the butyl alcohol was removed from the extract by distillation, using a vacuum pump, and the residue dissolved in ethyl ether. The ether-soluble fraction was added to olive oil, and the ether then removed by evaporation. This lot of urine yielded about 3000 R.U. of estrogenic substance to the liter.

Assay of estrogenic substance. The term estrogenic substance is used here to designate only those principles which produce estrous effects in animals whose ovaries have been removed. The unit was based on the vaginal growth effect in rats (15). The olive oil solution prepared from the butyl alcohol extract was assayed for estrogenic content by the technic of Coward and Burn (16). The quantity required to produce estrus in 50% of 20 ovariectomized, sexually mature, albino rats constituted the rat unit employed in both experiments.

On the basis of assays made with known quantities of crystalline estrone, it is estimated that the rat unit which we used was equivalent to approximately 3.4 I.U. This, however, is but a conventional value, and may have no real meaning in relation to the total physiological activity of the extract. It has been shown (17) that attempts to use an estrone standard to compare the activities of other excretory forms of the estrogenic hormones, or of crude extracts, are subject to many sources of error.

Animals. Sexually mature, male rabbits of a pure albino breed were used in both experiments. These were healthy animals from an inbred strain raised in this laboratory. They were kept in individual cages in a well ventilated room receiving light from two sides, and were fed

a uniform diet consisting of hay, Chinese cabbage (*pai ts'ai*), and steamed soya beans. No water was provided in addition to that contained in the food.

In the first experiment 16 animals ranging in age from 16 to 17 months were used. In the second experiment, 26 animals, 13 to 21 months of age were used. In each case the animals were divided into a control and a test group. At the time treatment was commenced none of the animals had been mated.

Inoculations. The strain of *Treponema pallidum* used was that originally isolated by Nichols. The immediate source of virus in each experiment was the testicular tissue from a rabbit with a progressing syphilitic orchitis. The animals in the first experiment were inoculated on April 15, 1934 with 0.3 cc. of a saline emulsion containing from 2 to 3 actively motile organisms to the microscopic field. The strain at that time had undergone 31 serial animal passages since it was brought to China in 1927.

The animals of the second experiment were inoculated on Oct. 15, 1935, with 0.3 cc. of a saline emulsion containing from 4 to 5 *Treponema pallidum* to the microscopic field. The organisms were actively motile. At that time they had undergone 38 serial animal passages in this laboratory. In each case inoculation was made into the right testis. Animals from the control and test groups were inoculated alternately so that no two animals from the same group received material from the same syringe.

Treatment with estrogenic substance. In the first experiment treatment was begun 15 days before inoculation. From 20 to 60 R.U. of estrogenic substance in olive oil was injected subcutaneously once a day, 6 days a week, into each of 8 rabbits. A second group of 8 animals were injected in like manner with the same quantity of plain olive oil. The urinary extract was prepared so that 1.0 cc. of the olive oil solution contained 30 R.U. of estrogenic substance.

In the second experiment, treatment had progressed for 76 days when the inoculations were made. In this case 30 R.U. of estrogenic substance was administered daily, 6 days a week, to 14 animals. Two of these animals died from a respiratory infection not long after inoculation, and are not included in the analysis of the results.

By combining the two experiments there was a total of 20 animals in each group. In the control group, which received plain olive oil injections, the animals were observed for a mean period of 199.5 $\pm$10.61 days after inoculation, and those in the test, or estrinized group were observed for a mean period of 188.2 $\pm$6.74 days.

Treatment in both experiments, although commenced at different times with respect to inoculation, was continued in all animals as long as they survived, or until the end of the experiment. Because of this difference in the relation of treatment to the duration of syphilis, the animals in the first experiment showed only the early physiological effects of the estrogens at the inception of syphilitic infection; while in the second experiment, the disease began in animals with far advanced signs of the feminizing effect of treatment.

Statistical methods. Throughout the paper all mean values are expressed together with their standard deviations. The statistical significance of the differences between mean values is expressed in many instances as the probable occurrence (*P*) of a deviation as great as or greater than the designated one in 100 trial samples from the same universe of data. A value of P of 0.01, or less, is considered significant, and a value of 0.04, probably significant.

For comparing the number of animals in any two groups, the Chi-square test for a fourfold table was applied, and for the comparison of the number of days in any two groups, the difference between the means and their standard deviations was used. The standard deviation of the mean was computed from the formula $\sigma/\sqrt{N-1}$.

When one of the two groups compared contained 10, or less animals, the significance of the deviation was tested by using the statistic *t*, and the value of *P* obtained by entering Fisher's table. This value was used in case it indicated a greater probability of the difference being due to random sampling than did the former method of testing.

An appraisal of the physiological effects of the estrogenic substance was made on the basis of the observed modifications either in the form or function of the following structures: the nipples and mammary glands; the external genitalia, including testis, scrotum, and penis; and on the development of dewlap. Changes in the psychosexual behavior of the estrinized rabbits were also observed. In cases where mammary secretion was sufficient, the response of the feminized male to the suckling of new-born rabbits was tested.

An evaluation of the response to infection was made upon the character and the chronological sequence of the clinical phenomena of disease. Particular attention was given to the following: *a*), the incidence and time of occurrence of primary orchitis and chancre, together with the magnitude and duration of the lesions at the site of inoculation; *b*), the incidence, time of occurrence, and magnitude of metastatic orchitis and chancre of the uninoculated testis and scrotum; *c*), the frequency, number, and time of occurrence of generalized metastatic lesions of the skin, bone and periosteum, and eyes; *d*), the number of animals with complete healing of all detectable lesions at the termination of the period of observation; *e*), the number of days of latency per animal; and *f*), the nature of the serological response to infection.

Blood for serological tests was drawn from the marginal ear veins of all animals at biweekly intervals during the entire period of observation. The following tests for syphilitic reagin were made: Wassermann reaction, using the standard Kolmer quantitative technic, the Kahn and Kline reactions. In each case the technic used was the same as that employed for testing human blood.

Throughout the period of observation all animals were weighed at weekly intervals. At the completion of the experiment, or upon the death of the animals, the following organs were removed and weighed, care being taken to prevent loss of weight through evaporation: anterior and posterior pituitary, thyroid, parathyroids, adrenals, seminal vesicles and prostate bulbourethral glands, testes, liver and spleen.

RESULTS

In describing the results obtained in the two experiments it is necessary to consider, first, the physiological action of the estrogenic hormones on the male rabbits, and, secondly, the character and course of the syphilitic infection as conditioned by the effects of estrogenic therapy.

The anatomical changes induced by treatment are illustrated in figures 1 to 14, and in table 1 (Part I); and the results of treatment on the course of the disease are recorded in tables 1 to 6, and in figures 1 and 2 (Part II).

Changes in Structure and Function Induced by Estrogenic Substance

Mammary glands. The first manifest effects of treatment were related to the nipples and the mammary glands. Enlargement of the former began almost at once, and by the 16th day of treatment had progressed to the size found in pseudopregnancy (fig. 2). At this stage a clear secretion could be expressed from the nipples of many animals. By the 80th day of treatment the nipples were approximately as large as those of a lactating female rabbit.

The only remarkable difference in the response to treatment between the two experiments was in the character of the mammary secretion. In 6 of 8 animals of the first experiment, the secretion after 80 days of treatment was thick and white like milk, and could be expressed in about 0.5 cc. amounts from a single nipple of some animals. The secretion from the rest of the treated rabbits was always thin and clear. After continuing for at least 90 days, secretion in all animals decreased even though treatment was still being given. Subsequently, milk could be expressed only from one or two nipples in most animals, and in the case of a single rabbit secretion stopped entirely after having been present for 120 days. Four rabbits continued to secrete a milky substance for as long as 200 days.

In the second experiment, no animal developed secretion as copious as in the first experiment. After 20 days of treatment, however, all animals were secreting a clear, thin fluid; and after 95 days, 11 of those treated with estrogen developed a turbid, milky secretion. This remained thin in character and small in amount. As in the first experiment, secretion again ceased at about 135 days following the initial injection.

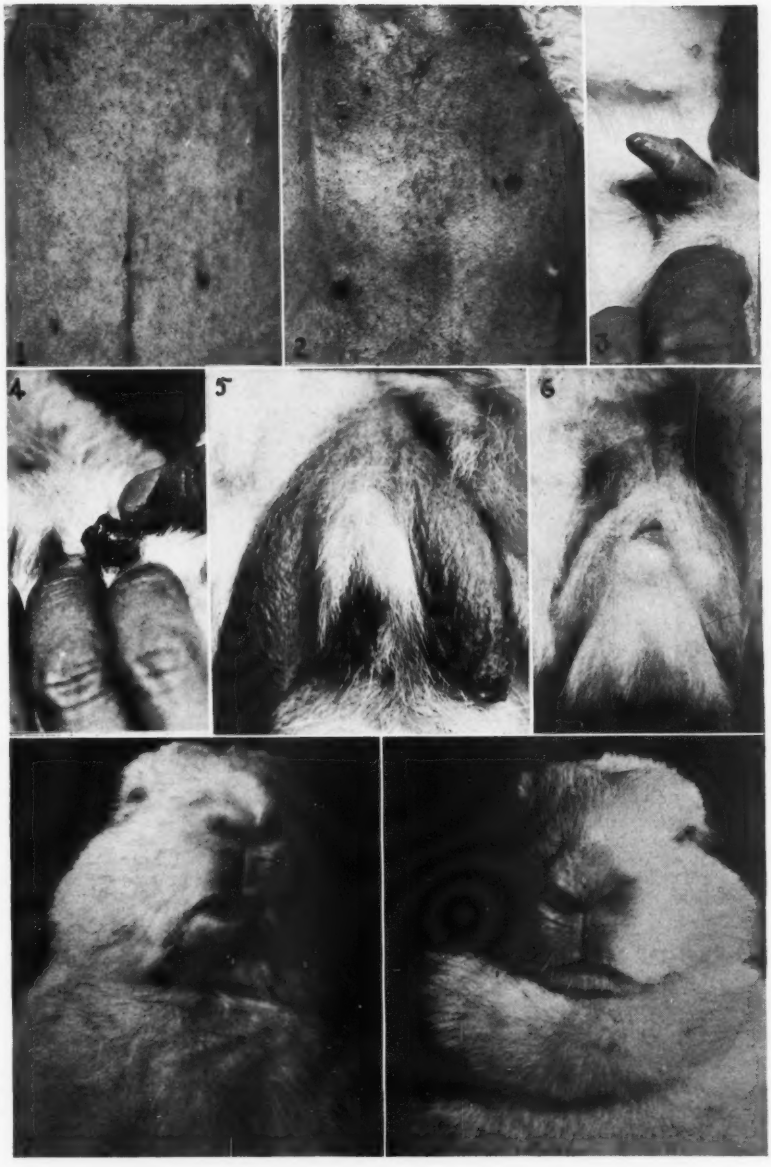

Fig. 1. Nipples of a normal adult male rabbit. Fig. 2. Hypertrophy of nipples of an adult male rabbit following administration of estrogenic substance. Fig. 3. Penis of normal adult male rabbit with prepuce retracted. Fig. 4. Atrophy of penis of adult male rabbit following administration of estrogenic substance over a period of 269 days. Fig. 5. Scrotal sacs of normal adult male rabbit. Fig. 6. Atrophy of scrotal sacs of adult male rabbit following administration of estrogenic substance for 118 days. The remnants of the sacs are covered with hair. The testes have partially ascended to the inguinal opening. Fig. 7. The neck of a normal adult male rabbit. Fig. 8. Formation of redundant skin of neck, known as dewlap, as it appeared following administration of estrogenic substance over a period of 192 days.

From time to time, however, small amounts of turbid fluid could be expressed from the nipples of 6 of these animals.

Histological examination of the mammary glands showed an extensive growth of the duct system, and in one animal from which the glands were removed during the most active phase of lactation, there was localized development of alveolar tissue. The extent of glandular development among the other animals is unknown as secretion had almost ceased when the glands were removed at the end of the experiment. However, in the actively secreting gland which was examined, the degree of growth

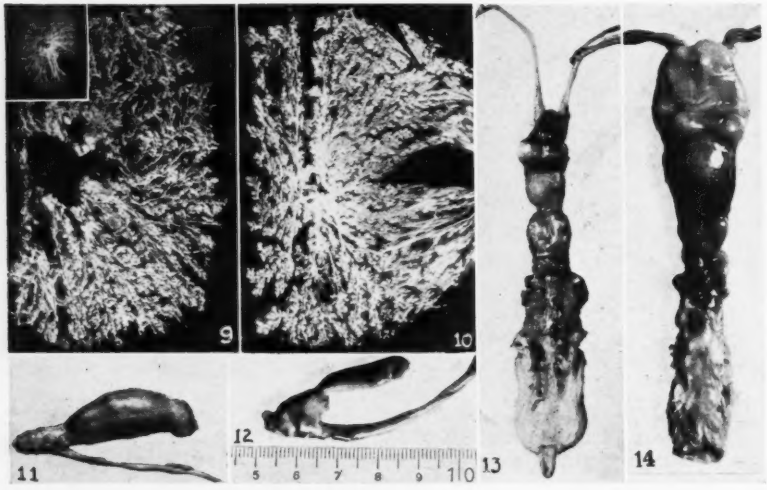

Fig. 9. Whole mounts of mammary glands taken from a normal male rabbit before (inset) and after treatment with estrogenic susbtance (30–60 r.u. daily for 185 days). Contact prints. Fig. 10. A whole mount of a mammary gland taken from an adult female rabbit on the 8th day of pseudopregnancy. Contact print. Fig. 11. Testis, epididymis and a part of the vas deferens from a normal adult male rabbit. Fig. 12. Atrophy of testis following prolonged administration of estrogenic substance (286 days). Fig. 13. Penis, bulbourethral gland, prostate and seminal vesicles of a normal adult male rabbit. Fig. 14. Atrophy of the penis to a form resembling the urogenital vestibulum of the female rabbit after 286 days of treatment with estrogenic substance. The seminal vesicles are greatly enlarged.

of the duct and alveolar systems was equivalent to that found in pseudopregnancy (fig. 9, 10).

External genitalia. A decrease in the size of both testes was appreciable as early as 30 days after treatment was commenced, but in most animals this change was not apparent before 80 days of treatment. In 9 animals treated over 114 days, the testes were less than half their original size and were held high in the scrotum at the inguinal ring. In this position some were fixed. With atrophy and ascent of the testes, the scrotal sacs shortened. The usual smooth surface of the dependent portion disappeared, leaving a shallow sac covered completely with hair (fig. 6). The recession of the scrotum was first detected after 44 days of treatment, and after 140 days the scrotum was no longer visible.

After 50 days of treatment the glans penis softened, and the urinary meatus began a process of elongation. Preceding these changes the prepuce appeared red and edematous for several days. Shortening of the glans progressed together with ventral

cleavage until the corpus cavernosum urethrae had receded into the depths of the prepuce. After 114 days of treatment the urethra could be everted and the mucosal surface rolled easily onto the outside of the shaft of the penis. By the time 150 days of treatment had elapsed the glans had disappeared with the exception of two lateral tags which were the remnants of the cavernous bodies. Later, these also disappeared. This left the atrophied cavernous portion of the glans penis tapering distally to a

TABLE 1. COMPARISON OF ORGAN WEIGHTS IN A GROUP OF RABBITS SUBJECTED TO PROLONGED ESTROGENIC TREATMENT WITH THOSE IN A GROUP OF UNTREATED RABBITS

Group	Anterior pituitary		Posterior pituitary		Whole pituitary		Thyroid		Parathyroids		Adrenals	
	N	Mean$\pm\sigma_m$	N	Mean$\pm\sigma_m$	N	Mean$\pm\sigma_m$	N	Mean$\pm\sigma_m$	N	Mean$\pm\sigma_m$	N	Mean$\pm\sigma_m$
		mg./kg.		mg./kg.		mg./kg.		mg./kg.		mg./kg.		mg./kg.
Control	18	7.78±0.43	18	2.48±0.17	18	10.66±0.56	17	186.4±34.8	18	9.57±0.96	18	274.8±21.4
Estrinized	19	8.42±1.99	19	2.42±0.27	20	11.00±0.54	19	109.9±17.1	19	7.84±0.77	20	249.3±52.2
Difference		+0.64±2.03		−0.07±0.32		+0.34±0.78		−76.5±38.7		−1.72±1.23		−25.5±56.3
P		0.757		0.834		0.67		0.048		0.165		0.753

N=number of animals. σ_m=standard deviation of the mean.

narrow band visible on the inner ventral surface of the prepuce. At this stage the prepuce, penis and urethra resembled the female pudenda with its clitoris and common urogenital vestibulum (fig. 4).

Skin and hair. The coats of the feminized males were in general heavier and cleaner than those of the animals in the control group. The difference was sufficiently striking

TABLE 2. COMPARISON OF ORGAN WEIGHTS IN A GROUP OF RABBITS SUBJECTED TO PROLONGED ESTROGENIC TREATMENT WITH THOSE IN A GROUP OF UNTREATED RABBITS

Group	Seminal vesicles		Bulbourethral gland		Testes		Liver		Spleen	
	N	Mean$\pm\sigma_m$	N	Mean$\pm\sigma_m$	N	Mean$\pm\sigma_m$	N	Mean$\pm\sigma_m$	N	Mean$\pm\sigma_m$
		mg./kg.		mg./kg.		mg./kg.		gm./kg.		gm./kg.
Control	18	526.9±64.7	17	189.5±22.5	17	1002.4±84.0	15	35.9 ±1.3	15	0.89±0.34
Estrinized	20	866.8±83.6	17	88.4±5.5	19	303.6±30.9	17	36.9±1.3	18	2.35±0.94
Difference		+339.9±105.7		−101.1±23.1		−698.8±89.6		+ 1.0 ±1.8		+1.46±0.10
P		<0.002		<0.002		<0.002		0.80		0.14

to make possible the identification of the members of the respective groups by this means alone.

Several animals of both groups developed subcutaneous abscesses at the site of injection. A thick grumous, oily exudate was evacuated from these lesions, which were apparently caused by the presence of olive oil in the tissues, and bore no relation to the estrogenic substance.

Dewlap, a secondary sexual characteristic of the female rabbit (18) was first visible from 30 to 90 days after the first injection of estrogen, and attained its full growth within 25 days after its presence became apparent (fig. 8).

Psycho-sexual behavior. The feminized males were more docile and passive than normal male rabbits. When placed with does they acted with indifference toward them and in no case attempted copulation. They fled from normal males that attempted copulation, and occasionally uttered sharp cries when attacked. In one case copulatory actions of a normal male were terminated by ejaculation, but no spermatozoa could be found in the vestibulum-like structure of the feminized male.

Young rabbits 8 days old were removed from their mother's cage and placed with lactating males. These animals the lactating males willingly fostered, and resisted their removal from the cage by vigorously attempting to bite our hands. In two instances the males suckled the young animals. The newly born rabbits survived from 5 to 6 days under such conditions, which was from 1 to 2 days longer than litter mates caged with normal males.

Autopsy. At the conclusion of the experiments autopsies were performed on 37 animals, of which 18 were in the treated group. Excluding the testes, syphilis, as a rule, does not produce any important lesions in the visceral organs of the rabbit. With this exception, such gross anatomical changes as were observed are attributed to the effect of the estrogenic substance.

Gross changes in structure were limited almost entirely to the testes (fig. 12) and accessory sex organs (fig. 14). Other viscera showed no important alterations. With the exception of the gonads and accessory sex glands, only the thyroid, among the organs of internal secretion which were examined, appeared altered in size. The mean relative weight of the thyroid removed from the estrinized animals was 76.6 ± 38.70 mg. per kg. less than that of the normal animals. This difference approaches probable statistical significance ($P = 0.048$). When compared with the differences between the two groups in the weight of other endocrine glands, including the pituitary, parathyroids and adrenals, it is apparent that the thyroid was the only one of these structures to show significant changes in weight (table 1).

There were important differences in the weights of the gonads and accessory sex organs (table 2). Hypertrophy of the seminal vesicles was evident from inspection of the organ (fig. 14). At autopsy, on opening the abdomen of some animals, the seminal vesicles were so much enlarged and distended with fluid as to be mistaken for the urinary bladder. In the rabbit the seminal vesicles and prostate are fused into a single structure. It was impossible, therefore, to separate the two in order to weigh the parts separately. It seemed, however, that the enlargement of the combined organ depended chiefly upon an increase in size of the vesicular portion.

The walls of the seminal vesicles looked thick and heavy. The mean difference between the relative weights of the combined prostate and seminal vesicles in the two groups was 339.9 ± 105.70 mg. per kg., a highly significant deviation ($P = < .002$). The bulbourethral gland was greatly decreased in size.

In considering the weights of the testes, it should be borne in mind that among the animals of the test group, these structures were subject not only to the effect of estrogens but also to the influence of syphilis. This disease of itself, when localized in the testis, can produce atrophic changes. The loss of testicular weight shown by the estrinized animals, therefore, might have been due in part to the action of both factors. In order to eliminate as far as possible the complication of a dual effect, we have compared the weights of the uninoculated testes of both groups. Of the animals in the control group, 17 developed metastatic lesions of the uninoculated testes, but in only one of the estrinized animals was there a metastatic orchitis. A comparison of the weights of the uninoculated testes of the two groups would therefore reveal the relative influence of syphilis and estrogen in the production of testicular atrophy. In the absence of orchitis, a reduction in weight of the testes of the estrinized animals would be attributable to estrogen, whereas a loss of weight among the normal animals would of course be the result of syphilis.

In the control group, the mean weight of the uninoculated testis was 496.7 ± 35.7 mg. per kg., and in the estrinized group, 152.8 ± 16.2 mg. per kg. This is a ratio of 3.08:1, the testis affected only by syphilis being on the average 343.9 ± 39.2 mg. per kg. heavier than one affected by estrogen alone. The difference is highly significant

($P = <0.0001$), indicating that estrogenic action was the more potent factor causing loss of testicular substance.

It may be seen in table 1 that the ratio of the mean combined testicular weight between the normal and estrinized animals of both experiments was 3.03:1. This is almost the same distribution of weight as occurred where the testis was not the site of a syphilitic lesion. From this it is concluded that, in general, the loss of testicular weight was due chiefly to the action of the estrogens.

The absence of syphilitic lesions in these structures probably bore a significant relation to the loss of testicular substance. A knowledge of the specific nature of the anatomical changes within the testis would therefore identify the part of the organ which is concerned in the furtherance of syphilitic infection in the testis.

DISCUSSION

In general the changes induced were progressively in the direction of feminization. The response of different tissues and organs in a succession of orderly changes may be interpreted as indicating a variation in the threshold of susceptibility, dependent, perhaps, upon the degree to which pituitary function was stimulated or depressed by the estrogens.

The prominent anatomical alterations observed among the estrinized animals were related for the most part to the genital organs and to the mammary structures. The external genitalia were reduced to a form resembling that of the female rabbit. The involution of the scrotum and partial ascent of the testes returned the animal to a sexually neutral condition approaching that of early life.

The changes in the mammary structures were equivalent to those induced in normal female rabbits by pseudopregnancy. There was enlargement of the nipples, and growth of the primary ducts and rudimentary glands (fig. 9). Stimulation of a secretion grossly indistinguishable from milk occurred especially in the animals of the first experiment.

Similar nipple and mammary growth has been produced in some species by the action of estrone and estriol. However, only in the guinea pig, of the species extensively studied, has estrone been observed to cause complete growth of the mammary glands (19, 20).

Although the development of the mammary glands is dependent primarily upon the action of estrogen (21), the initiation and maintenance of lactation has been shown to be a function of the anterior pituitary (22, 23). The appearance of milk in many of the male rabbits of these experiments demonstrated that the estrogens, or some other substance present in the urinary extract, influenced secretion, either directly, or by stimulating the release of the lactation-producing factor of the anterior pituitary. However, the prolonged administration of estrogenic substance exerted the opposite effect, as secretion ceased or became irregular during the latter part of the period of treatment. Besides, in the second experiment, the extract failed to induce the same quality of secretion as it had in the first.

Although there was considerable individual variation in the rate and degree of response to the estrogens, the difference between the animals of the two experiments with respect to lactation was too great to be due to chance alone. Whether or not ageing of the extract was a factor influencing this difference would require investigation. This possibility is suggested because of the time elapsing between the two experiments, approximately 14 months. As against this, is the absence of any other important difference in the response to treatment in the two experiments. The age and breed and general care of the rabbits in both cases were essentially the same. There remains, however, the possibility that the difference in the size of the dose of

estrogen was sufficient to account for the variation in the character and amount of secretion in the two experiments. It has been suggested that small quantities of estrogen affect the anterior pituitary in such a way as to promote the secretion of the lactation factor. In this connection it has been shown that the administration of large doses of estrogen results in a decrease or total disappearance of mammary secretion in the rabbit (24). As pointed out by Zondek (25), whether estrogen activates or depresses the function of the anterior pituitary depends upon the amount and duration of action of the hormone; small doses stimulate, while large doses depress.

Kemp and Shaw (26), who administered daily 20 I.U. of estrone in oil to adult male and female rabbits for 147 days found "slight mammary enlargement" at the end of this time, but none of the other effects produced by the urinary extract we employed. Nevertheless, among the animals treated with estrone there was apparently a reduction in the frequency of generalized lesions of a syphilitic infection. The disease in this case was induced by inoculating granulating wounds of the back 28 days after the beginning of treatment.

Enlargement of the seminal vesicles with hypertrophy of the muscular walls such as occurred among the animals of these experiments has been produced in rats, mice and monkeys following administration of estrone (27). Atrophy of the gonads and accessory sex glands has been induced in laboratory animals by the same substance (28).

The mode of action of the estrogens on the gonads and accessory sex organs is attributed to their depressant effect on the anterior pituitary, resulting in the inhibition of the secretion of the gonad-stimulating principle. In this connection, it is to be noted that the weight of the component parts of the pituitary among the animals of these experiments was not affected by estrogenic treatment (table 1). As the size of an organ may tell nothing about its functional activity, further study is required for evidence of cytological changes.

The lower weight of the thyroid, and to a less extent of the parathyroids and adrenals, suggests that these structures were affected in much the same way as the testes.

Another important property of the extract was its ability to cause the growth of a dewlap in all of the treated male rabbits. This peculiar fold of redundant skin, which encircles the anterior neck, occurs normally in about 50% of the female rabbits in our breeding colony after they have attained the age of sexual maturity. It was found that the stimulating effect of the estrogens in this connection depended upon an integrating factor which was present only in the adult rabbit, as dewlap could not be induced artificially in animals of either sex before the age of 6 months. The final evidence of the exact sexual relationship of the dewlap was furnished by the finding that its growth and maintenance in normal female rabbits depended upon the presence of ovarian tissue (18).

Conclusions. These observations provide important reasons for concluding that the urinary extract we prepared contained a substance, or substances, physiologically identical, at least in several respects, with the internal secretion of the ovary. Some of the effects obtained through the use of the extract have not been observed after the administration of the pure crystalline hormones, estrone and estriol. However, the action of these substances on the rabbit has not been extensively studied, especially when administered over a long period of time. Furthermore, the crystalline estrogens already isolated may not constitute the whole of the estrogenic hormone contained in the extract employed in these experiments.

REFERENCES

1. Colles, A.: Practical Observations on the Venereal Disease, London, 285. 1837.
2. Hutchinson, J.: *Med. Times and Gazette* 2: 643. 1876.
3. Brown, W. H., and L. Pearce: *Am. J. Syph., Gonor. & Ven. Dis.* 4: 593. 1920.
4. Chesney, A. M.: *J. Exper. Med.* 38: 627. 1923.
5. Kemp, J. E.: *J. Infect. Dis.* 60: 32. 1937.
6. Moore, J. E.: *Bull. Johns Hopkins Hosp.* 33: 231. 1922.
7. Moore, J. E.: *Bull. Johns Hopkins Hosp.* 34: 89. 1923.
8. Moore, J. E.: *Arch. Int. Med.* 30: 548. 1922.
9. Solomon, H. C.: *Am. J. Syph., Gonor. & Ven. Dis.* 10: 96. 1926.
10. Menninger, W. C., and J. E. Kemp: *Ann. Int. Med.* 9: 175. 1935.
11. Frank, R. T., M. L. Frank, R. G. Gustavson and W. W. Weyerts: *J.A.M.A.* 85: 510. 1925.
12. Aschheim, S.: *Arch. f. Gynäk.* 132. 179. 1927.
13. Frazier, C. N., J. W. Mu and C. K. Hu: *Proc. Soc. Exper. Biol. & Med.* 33: 65. 1935.
14. Veler, C. D., S. Thayer and E. A. Doisy: *J. Biol Chem.* 87: 357. 1930.
15. Long, J. A., and H. M. Evans: *Memoirs University of California* 6: 1. 1922.
16. Coward, K. H., and J. H. Burn: *J. Physiol.* 63: 270. 1927.
17. Emmens, C. W.: *Great Britain Med. Research Council, Special Rep. Series*, No. 234, 1939.
18. Hu, C. K., and C. N. Frazier: *Proc. Soc. Exper. Biol. & Med.* 38: 116. 1938.
19. Smelser, G. K.: *Physiol. Zoöl.* 6: 396. 1933.
20. Turner, C. W., and E. T. Gomez: *Missouri Agricultural Exp. Station Research Bull.*, 206, 1934.
21. Turner, C. W., A. H. Frank, W. U. Gardner, A. B. Schultz and E. T. Gomez: *Anat. Rec.* 53: 227. 1932.
22. Stricker, P., and F. Grueter: *Presse med.* 37: 1268. 1929.
23. Corner, G. W.: *Am. J. Physiol.* 95: 43. 1930.
24. Smith, G. V. S., and W. Smith: *Am. J. Physiol.* 103: 356. 1933.
25. Zondek, B.: *Lancet* 2: 842. 1936.
26. Kemp, J. E., and C. Shaw: *Am. J. Syph., Gonor. & Ven. Dis.* 22: 9. 1938.
27. van Wagenen, G. *Science* 81: 366. 1935.
28. Moore, C. R., and D. Price: *Am. J. Anat.* 50: 13. 1932.

INCREASED RESISTANCE TO SYPHILIS IN THE RABBIT FOLLOWING PROLONGED ADMINISTRATION OF URINARY ESTROGENS

II. CHARACTER OF THE REACTION TO *TREPONEMA PALLIDUM* IN FEMINIZED MALE RABBITS

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IN THE PRECEDING PAPER (1), the feminizing effects of an estrogenic extract of human pregnancy urine were described as they occurred in adult male rabbits. Two experiments were undertaken. In the first of these, from 20 to 60 R.U. of estrogenic substance were administered daily for 15 days before the animals were inoculated with *Treponema pallidum*. In the second experiment, the animals were under estrogenic treatment for 76 days (30 R.U. daily) before the inoculations were performed. In all animals, the inoculations were unilateral into the right testis.

With the exception of the primary testicular and scrotal reactions to infection, it is unnecessary to describe separately the results of the two experiments as otherwise the disease followed parallel courses. Such divergencies as were observed came early in the disease and were dependent, apparently, upon the duration of treatment at the time of inoculation. Therefore, the animals have been combined into a control and a test group, each comprising 20 rabbits. As the animals of the control group were sexually intact, they will be referred to as normal rabbits, and those of the test group, as the treated or estrinized rabbits.

As the chief differences in the disease to be described consist of variations involving the incidence of certain phenomena of infection, and the time relationships of their sequence and duration, a comparison of the two groups has been made on the basis of the respective combined mean values (tables 1 to 6). The number of animals is sufficiently large in most cases to give the statistical comparisons a considerable degree of reliability.

OBSERVATIONS

Primary orchitis. It may be seen in table 1 that all animals in the control group developed a primary orchitis, but only 16 of the 20 estrinized animals showed a similar reaction in the inoculated testis. Those animals that failed to respond with an orchitis belonged to the second experiment and had been subjected to prolonged treatment (76 days) before inoculation. Almost without exception an orchitis may be expected to develop in the testis of a rabbit inoculated with the Nichols strain of *Treponema pallidum*. For this reason alone, the refractory state of the testis shown by 4 of the treated rabbits is noteworthy. Furthermore, statistical analysis showed that the variation in the incidence of orchitis was probably not due to chance ($P=0.036$).

With a single exception, the treated animals that did not present a primary orchitis developed a scrotal nodule which later ulcerated to form a chancre. In the ani-

Received for publication August 2, 1940.

TABLE 1. DIFFERENCES BETWEEN ESTRINIZED AND NORMAL MALE RABBITS IN THE INCIDENCE OF THE PHENOMENA OF SYPHILITIC INFECTION

Group and differences	Primary orchitis		Primary chancre		Metastatic orchitis		Metastatic chancre		Generalized lesions		Bone and periosteum		Skin		Eye		Complete healing	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Control N=20	20	100	11	55	15	75	8	40	17	85	15	75	9	45	5	25	5	25
Estrinized N=20	16	80	14	70	1	5	4	20	10	50	6	30	1	5	4	20	15	75
X^2		4.44		0.96		20.42		1.90		5.58		8.12		8.53		0.14		10.0
P		0.036		0.327		0.0001		0.168		0.018		0.004		0.003		0.709		0.002

N=Number of animals.
X^2=Chi-square for a four-fold table.
P=Probability of the difference being due to random sampling; value of 0.04 is considered probably significant, and a value of 0.01, or less, significant.

mal that had neither orchitis nor chancre the infection remained continuously latent as the animal did not show any demonstrable lesion, primary or metastatic, during the entire period of observation. The presence of *Treponema pallidum* in the tissues of this rabbit was demonstrated at the conclusion of the experiment by the transfer of popliteal lymph nodes into normal rabbits.

In describing syphilitic lesions of the testis and scrotum it is customary to consider them as a unit because they seldom remain separate processes, but merge to form a single inflammatory lesion. In these experiments, however, it was only the exceptional animal among those treated with estrogen that showed this type of reaction. Instead, the testicular lesion remained localized, and was often confined to one segment of the testis. There was in no case a reaction of critical edema with involvement of the tunica to form a swollen and indurated mass. When the scrotum developed a lesion, it began as a small solitary nodule of the surface of the sac, which, even though it enlarged to form a chancre, did not join the testicular lesion. Orchitis began in a very limited area as a small, well circumscribed infiltration which grew slowly but never reached a very large size. The reaction of the normal rabbits as far as the testis is concerned, was of average severity and followed a normal course.

The primary testicular response of the treated animals in the two experiments did not follow the same pattern. In the first experiment where the rabbits had been under treatment for only 15 days at the time of inoculation, the degree of testicular reaction was greater. In fact, the size of the involved testes was somewhat larger than in the control animals. The incubation period of primary orchitis among the estrinized rabbits of the first experiment was shorter than among the normal rabbits. The differences, both in degree of reaction and in rapidity of onset, suggest a transient initial increase in testicular susceptibility at the beginning of estrogenic treatment.

TABLE 2. DIFFERENCES BETWEEN ESTRINIZED AND NORMAL MALE RABBITS IN THE INTERVALS OF TIME ELAPSING BETWEEN INOCULATION AND THE ONSET OF THE VARIOUS PHENOMENA OF SYPHILITIC INFECTION

Group	Primary orchitis		Primary chancre		Metastatic orchitis		Metastatic chancre		Bone		Skin		Eye	
	N	Mean±σ_m	N	Mean±σ_m	N	Mean±σ_m	N	Mean±σ_m	N	Mean±σ_m	N	Mean±σ_m	N	Mean±σ_m
		days		*days*		*days*		*days*		*days*		*days*		*days*
Control N=20	20	22.9±1.8	11	81.9±14.5	15	78.6±9.7	8	94.0±14.3	15	111.7±11.8	9	109.5±16.7	5	126.2±13.2
Estrinized N=20	16	27.9±3.7	14	43.0±3.4	1	59.0	4	88.7±23.0	6	136.0±25.0	1	115.0	4	164.0±0
Difference		+5.0±4.1		−38.9±14.9		−19.6[1]		−5.3±27.1		+24.3±27.6		+5.5		+37.8±13.2[2]
P		0.231		0.009		0.7–0.6		0.849		0.379		0.90		0.05–0.02

N=number of animals. σ_m=standard deviation of the mean. [1] t=0.51 [2] t=0.1040

TABLE 3. EFFECT OF THE DURATION OF ESTROGENIC TREATMENT ON THE PRIMARY RESPONSE TO INFECTION WITH TREPONEMA PALLIDUM

Group	Incubation period of primary orchitis				Difference	P
	Exper. I[1]		Exper. II[2]			
	N	Mean ± σ_m	N	Mean ± σ_m		
		days		days		
Control	8	20.4 ± 2.6	12	24.7 ± 2.5	+4.3 ± 3.6	0.234
Estrinized	8	16.4 ± 1.2	8	39.5 ± 4.4	±23.1 + 4.6	0.0001
Difference		−4.0 ± 2.9		+14.8 ± 5.0		
P		0.171		0.003		

[1] Treated for 15 days before inoculation.
[2] Treated for 76 days before inoculation.

The mean incubation period of the infection (table 2) among the normal animals of the combined experiments was 22.9 ± 1.8 days, and among the estrinized animals, 27.9 ± 3.7 days. Although this difference is too small to be of statistical importance, the longer incubation period among the treated animals may be taken as the first indication of the inhibitory effect of the estrogenic hormones on the course of disease.

The importance of this difference is brought out by a study of the chronological sequences in each of the two experiments. This shows the relation of the duration and amount of treatment to the early progress of the disease. The inhibitory influence of the estrogens on the initial response to infection was manifest only in the last experiment, in animals treated over a period of 76 days before inoculation. In the first experiment, as stated above, there was an acceleration rather than a retardation of the primary reaction in the inoculated testis. This variation was evidently the result of the duration of treatment and probably depended upon differences in the degree of testicular involution due to estrogenic activity.

The variations with respect to the mean time of occurrence of orchitis under the different circumstances of treatment are summarized in table 3. From this table it may be seen that the estrinized rabbits in the second experiment had a significantly longer period of incubation than did the normal animals. The mean difference per animal was 14.8 ± 5.0 days.

The effectiveness of the estrinized animals' defensive reaction is well shown by the rate of resolution of the testicular lesions (table 4). This was about the same among the animals of the two experiments. The mean duration of primary orchitis in the normal animals of the combined experiments was 125.1 ± 12.4 days, but in the estrinized animals it was only 48.1 ± 7.9 days. The mean difference of 76.9 ± 14.7 days affords a good demonstration of the relative testicular resistance to the disease which was developed by the treated rabbits.

TABLE 4. DIFFERENCES BETWEEN ESTRINIZED AND NORMAL MALE RABBITS IN THE DURATION OF THE VARIOUS PHENOMENA OF SYPHILITIC DISEASE

Group	Primary orchitis		Primary chancre		Metastatic orchitis		Metastatic chancre		Bone		Skin		Eye	
	N	Mean ± σ_m	N	Mean ± σ_m	N	Mean ± σ_m	N	Mean ± σ_m	N	Mean ± σ_m	N	Mean ± σ_m	N	Mean ± σ_m
		days		days		days		days		days		days		days
Control N=20	20	125.1 ± 12.4	11	76.0 ± 19.9	15	62.9 ± 12.5	8	64.3 ± 15.2	15	29.5 ± 5.5	9	15.1 ± 3	5	30.8 ± 13.8
Estrinized N=20	16	48.1 ± 7.9	14	77.2 ± 14.3	1	42	4	38.5 ± 24.5	6	38.5 ± 11.5	1	91	4	24.5 ± 8.8
Difference		−77.0 ± 14.7		+1.2 ± 24.5		*		−25.8 ± 28.8		+9.0 ± 12.8		**		−6.3 ± 16.4
P		<0.0001		0.960		0.9–0.8		0.373		0.484		0.01		0.704

* $t = 0.1317$ ** $t = 7.9979$

In the first experiment, at the time healing was complete, mammary secretion was at its point of maximal intensity. In the second experiment, mammary secretion was established before the inoculations were made. However, at the time the testicular lesions healed, the testes had become atrophic as a result of estrogenic treatment. This was approximately 75 days after inoculation.

Primary scrotal chancre. Whereas the estrinized animals developed a significantly lower incidence of orchitis in the inoculated testis, they reacted with a higher incidence of lesions of the scrotal sac (table 1), but the difference in this instance was not large enough to be given much weight. The onset of scrotal lesions came earlier in the treated animals, a reaction which was, in point of time, the reverse of the primary orchitis. The shorter incubation period of the scrotal chancre is of interest and might

TABLE 5. TIME OF OCCURRENCE OF THE TESTICULAR AND SCROTAL LESIONS IN EXPERIMENT 2. EFFECT OF ESTROGENIC TREATMENT ON THE REACTION TO INFECTION

Group	Incubation period						Difference	P
	Primary orchitis		Primary chancre		Metastatic orchitis			
	N	Mean $\pm \sigma_m$	N	Mean $\pm \sigma_m$	N	Mean $\pm \sigma_m$		
		days		*days*		*days*		
Control	12	24.7±2.5	4	104.5±26.9			79.8±27.0	0.003
Control			4	104.5±26.9	9	84.7±14.1	19.8±30.4	0.516
Estrinized	8	39.5±4.4	10	39.4±3.9	0		0.1±5.9	0.992

have been dependent upon the mildness of the preceding orchitis. This possibility we shall discuss later.

In this connection, it is generally believed (2, 3) that the course of syphilis in the rabbit is determined largely by the intensity and extent of the reaction developed during the preceding stages of the disease; in the event of an orchitis of severity and long duration, the following phases of infection are likely to be mild, while in the case of a mild and transient primary orchitis, the subsequent generalized manifestations of disease tend to be widespread and destructive.

From the study of the results of the combined experiments alone, certain significant differences in the relationships of the early manifestations of disease are overlooked. These are brought out, however, by an analysis of the differences in the time of occurrence of the testicular and scrotal reactions in the second experiment. The results of this comparison (table 5) throw some light on the relationship of the primary and metastatic phases of the disease, and also on the effect of estrogenic treatment upon these relationships.

We have made the comparisons in table 5 for the purpose of locating the position of the primary scrotal chancre in the course of the disease. By this device it is hoped to identify the scrotal lesion with either the primary or metastatic reaction. The onset of metastatic orchitis is used because it is the earliest clinical lesion identifying the generalization of the infection.

From the course of the disease in the normal animals, it is apparent that the primary orchitis and the primary scrotal chancre, in point of time, belonged to different stages of the infection. A period of 79.8 ± 27.0 days elapsed between the appearance of orchitis and chancre. This interval is too long to account for both lesions on the basis of a primary reaction at the site of inoculation. The fact that each lesion originated as a distinct focus is further evidence in support of this conclusion. Moreover, the mean time of occurrence of the primary chancre corresponds closely to that of the metastatic orchitis. This close association in time of onset of the two reactions is substantial proof that the primary scrotal lesion was metastatic in origin.

If, however, the same reasoning is applied to the orientation of primary chancre in the disease of the estrinized rabbits, it will be found to occupy a different position. In this instance the chancre and orchitis occurred simultaneously, there being a mean difference in their incubation periods of only 0.1 ± 5.9 days. The scrotal nodule preceding the chancre must then have appeared at the site of inoculation, and the chancre that followed must have been a primary lesion in the strict meaning of the

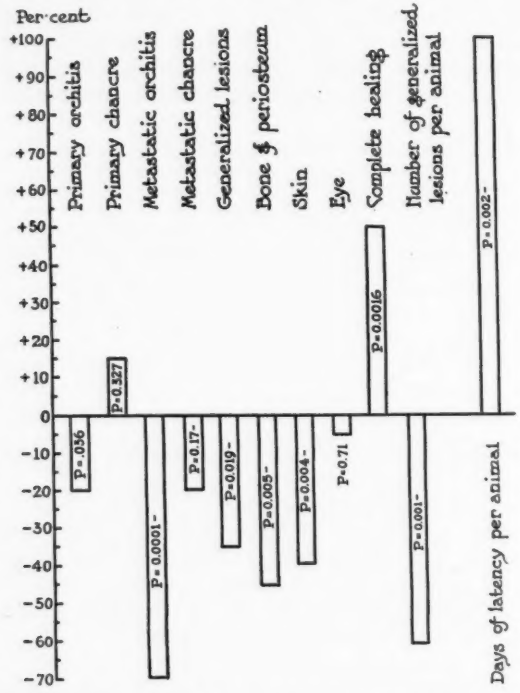

Fig. 1. INCIDENCE OF VARIOUS PHENOMENA OF SYPHILITIC DISEASE IN ESTRINIZED MALE RABBITS AS COMPARED WITH NORMAL MALE RABBITS. The variation is calculated as percentage deviation from the values observed among the normal animals. The statistical significance of the deviation is indicated by the value of *P*.

word, and not a metastatic focus as in the normal animals. On the basis of this reasoning, the assumption that the higher incidence of scrotal lesions in the treated animals was due to the mildness, or complete absence of orchitis does not seem valid.

Nevertheless, that there existed some reciprocal relationship between the immune responses of the two structures (testis and scrotum) which was affected by the action of the estrogens is suggested by the following observations. In the second experiment, all of the 12 normal animals developed a primary orchitis, but only 4 had a scrotal chancre; while among the 12 estrinized rabbits, 8 developed a primary orchitis, but 10 reacted with a chancre of the scrotum.

Regardless of the differences in time of onset of the scrotal lesions, they remained active as long in the estrinized as in the normal rabbits. This raises the question of the actual duration of all lesions at the site of inoculation. In spite of the lower incidence, mildness, and shorter duration of primary orchitis among the treated rabbits, it is possible that with the higher incidence of scrotal chancres, the actual time the tissues of testis and scrotum were the site of an active syphilitic process might have been

as great as, or greater than in the untreated rabbits. The compensatory position of the scrotal reaction under such circumstances might have exerted an important influence in equalizing the severity of the later course of the disease in the two groups.

For this reason we have compared the animals in the combined experiments with respect to the actual length of time there were active foci of disease in these structures. In the computation, the element of overlapping occasioned by the presence at the same time of orchitis and scrotal chancre was eliminated. In the control group there were 20 rabbits with primary lesions, and in the treated group, 19 animals with lesions of either testis or scrotum. The mean duration of active primary lesions in the former group was 185.3 ± 14.8 days, and in the estrinized group, 116.2 ± 12.6 days. The difference of 69.1 ± 19.4 days is well beyond the probability of random sampling ($P = 0.0003$). In other words, considering the total primary reaction in testis and scrotum, the estrinized animals in all respects thus far discussed had a significantly milder disease than the normal rabbits.

There remains but one more aspect of the scrotal reaction which enters into the total primary response, namely, the magnitude of the chancre. In general the area of scrotum involved, both by infiltration and by ulcer, was greater in the estrinized animals. These lesions did not involve the testis, but invaded only the scrotal sac and other extratesticular tissues. The freedom of the testes was demonstrated at autopsy by the absence of adhesions and deformity of the glands.

The trend of the primary reaction in the testis and scrotum is of interest in that it demonstrated clearly that the inhibitory influence of the estrogens on the disease did not affect equally the tissues of the testis and scrotum. Figure 2 shows the relationships between the time intervals of the primary reaction in these structures.

Metastatic orchitis. The inhibition of the reaction in the inoculated testis was the most conspicuous modification of the initial response to infection. However, an even more refractory condition of the uninoculated testis was manifest by almost complete suppression of metastatic orchitis. Only one of the estrinized rabbits developed a metastatic lesion of the testis, an incidence of 5%, whereas 15, or 75% of the normal animals developed similar lesions.

Although metastatic chancres of the scrotum among the estrinized rabbits were more frequent than metastatic orchitis, still their actual number was less than among the normal animals (table 1).

The absence of metastatic orchitis indicates either that a highly efficient general immunity had been established as a result of the primary reaction or that estrogenic treatment had conditioned the testis so as to make them no longer a favorable medium for the survival and multiplication of the infecting organisms. The failure of the infection to establish metastatic foci in the testis was perhaps the most noteworthy variation of the disease among the estrinized rabbits. As an organ the testis is highly susceptible to syphilis, and, under normal circumstances, is frequently the site of metastatic lesions.

Generalized lesions. The presence or absence of generalized lesions of syphilis in the rabbit is the most important single criterion of the effectiveness of the immunity established during the early course of the infection. As already pointed out, following an intense and prolonged testicular and scrotal reaction, it is usual to find the later period of the disease relatively free of metastatic foci. But in those animals poorly protected by minor testicular reactions or by suppression of the initial reaction, the succeeding generalized lesions are usually severe both as to the frequency of their occurrence, and to their intensity and extent.

As may be seen from figure 2, which illustrates the progression of infection, the stage of generalized lesions, with foci in the bones and periosteum, skin, and eye, had its inception among the normal animals while the primary and metastatic reactions in the testes were still present. This overlapping of generalized and testicular

stages was not as common among the estrinized animals. Instead, there was frequently a period of latency between the early and generalized reactions, when there were no demonstrable lesions.

A comparison of the tendency to overlap in the two groups of animals is of interest as it provides additional evidence of the extent to which treatment modified the course of syphilis. There were 17 normal animals and 10 estrinized animals with gen-

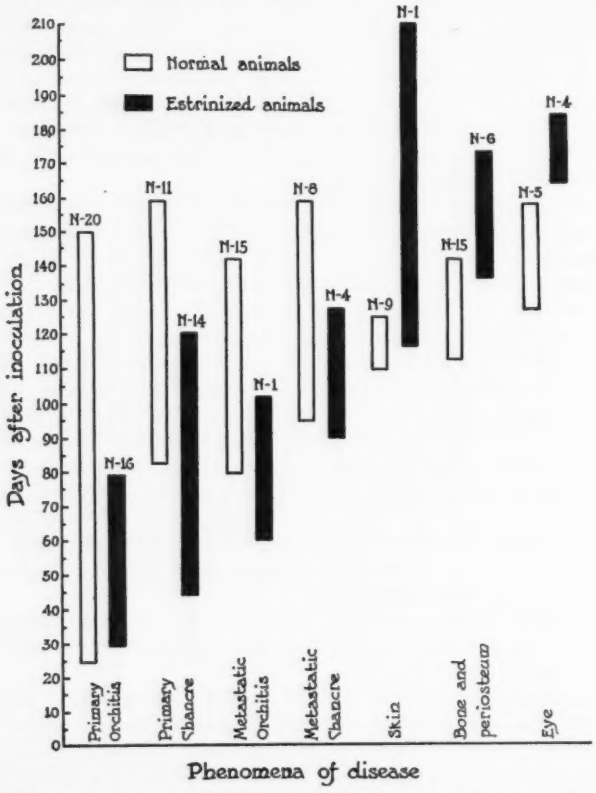

Fig. 2. Comparison of the progress of syphilitic infection in 20 estrinized and 20 normal male rabbits as determined by the time between inoculation and the onset and termination of the phenomena of disease. The position of the bar in each case is based on the mean number of days for the group, the number of animals in each group responding with the particular lesion being indicated by the figure at the top of the bar.

eralized lesions. The mean length of the period of overlapping among the normal rabbits was 55.1 ± 6.5 days, but among the estrinized animals it was a negative quantity, namely, -18.9 ± 24.0 days. The mean difference, expressed as an algebraic sum, is ~~4.6~~ 7.40 ± 24.9 days. This is an exceptionally large deviation considering the short course of syphilis in the rabbit.

Retardation in the appearance of the generalized phenomena of infection was common to the reactions occurring in each of the structures involved, being greatest in case of the eye (table 2). This accounted for part of the difference in overlapping, but most of it was due to the very short duration of the period of primary and metastatic orchitis among the estrinized animals.

Because of the wide differences in the severity and duration of the testicular reac-

tions in the two groups, it might be expected that the subsequent generalized phase of infection would reflect this divergency. If such had been the case, the milder and more transient early reactions of the estrinized animals would have been followed by a more severe form of disease than occurred among the normal animals. This was not the observed course of events.

The incidence of generalized lesions among the treated animals was significantly lower than among the normal animals. In the former group, 50% developed reactions in bone, skin, and eye, while 85% of the untreated animals had reactions of this nature. The greater susceptibility of the normal animals to the generalized effects of the disease is also evident from the number of demonstrable foci of infection each animal developed. Not only fewer of the feminized males reacted with generalized lesions, but those that did had fewer lesions per animal.

Of the 17 untreated rabbits presenting generalized lesions, there was a mean number of 3.6 ± 0.65 per animal, but among the estrinized animals with similar foci, there were only 1.4 ± 0.01 lesions to the rabbit. This is a highly significant difference (2.20 ± 0.014; $P = 0.0008$), which together with the relatively low incidence of generalized lesions among the treated animals should be given considerable weight in measuring the relative susceptibility to disease.

The character and location of the generalized lesions may also be used for a comparison of the differences in reaction between the two groups. Table 1 shows that the estrinized animals developed lesions of the bones and skin much less frequently than did the animals in which the disease was allowed to follow its natural course. Out of the 20 treated animals only one presented a lesion of the skin. This lesion was unusual in that it was located at the base of the penis and took the form of a chronic indurated ulcer. Keratitis appeared with equal frequency in both groups, but at a later time among the treated animals. The duration of the generalized lesions did not vary with treatment, if the chronic metastatic ulcer of the penis is excepted. This was present for 91 days (table 4). The mean duration of lesions of the skin in the control group was 15.1 ± 3.0 days.

In summarizing the effects of treatment observed during this stage of the disease, it may be stated, that although a definite retardation occurred in the time of onset of the generalized reaction among the estrinized animals, the only important deviations between the two groups bearing statistical significance were in the incidence of generalized lesions, and in their distribution among the animals affected. In these two respects, the effect of the estrogens was to inhibit the infection, and in some cases to suppress it entirely.

Period of latency. The duration of latency in syphilis may be taken as a measure of the effectiveness with which the host reacts to suppress infection. It constitutes the negative complement to the active phases of the disease already described. One of the estrinized animals of the second experiment carried an asymptomatic infection throughout the entire period of observation, a matter of 167 days. In our experience with the Nichols strain of *Treponema pallidum*, we cannot recall having seen another instance in which this organism failed to produce symptomatic disease in the rabbit after testicular inoculation with an inoculum of the size we were using.

A comparison of the two groups with respect to the duration of latency is of interest, and demonstrates the advantage possessed by the estrinized animals in controlling the disease. All of the animals in both experiments were used for determining the mean period of latency in each group regardless of the variation in the length of time they were observed. This does not introduce an important factor of error as the mean period of observation in the two groups differed only by 11.3 ± 12.6 days. Among the normal animals the mean duration of the latent period was 46.0 ± 7.8 days per animal. In the animals treated with estrogenic substance, this period was 92.0 ± 12.8 days per animal, a difference of 46.4 ± 15.0 days ($P = 0.002$). Thus it may

be seen that treatment acted to reduce by half the period of active syphilitic disease.

Recovery. A final comparison of the differences in the course of the disease may be made on the basis of the proportion of animals in each group with complete healing and resolution of lesions at the termination of the period of observation. The estrinized animals were observed for a mean period of 199.5 ± 10.6 days after inoculation, and the normal animals for 188.2 ± 6.7 days. At the expiration of this time 25% of the normal rabbits and 75% of the estrinized rabbits were free of clinical lesions. The more rapid recovery from active signs of disease is indicative of a more efficient immunologic reaction among the feminized male rabbits, and is in harmony with the preceding course of infection in these animals.

Serological reactions. Serological tests for syphilis were performed only on the rabbits of the first experiment. Reference to the details of the behavior of the reactions in each of the three tests used need not be made in this report. It is sufficient

TABLE 6. COMPARISON OF BODY WEIGHTS OF ESTRINIZED AND UNTREATED SYPHILITIC RABBITS

Group	Body weights				Difference	P
	Before treatment		After treatment			
	N	Mean ± σ_m	N	Mean ± σ_m		
		gm.		gm.		
Control	18	2526.7 ± 6.0	18	2383.9 ± 75.6	−142.8 ± 75.6	0.059
Estrinized	20	2529.0 ± 72.3	20	2525.0 ± 80.8	−4.0 ± 108.4	0.968
Difference		+2.3 ± 72.6		+141.1 ± 110.5		
P		0.976		0.20		

to observe here that there were no important differences between the two groups of animals in any of the reactions. Among the normal rabbits the tests became positive from 27 to 35 days after inoculation, and remained positive for from 61 to 117 days, depending upon the particular test. The Wassermann reaction was the least sensitive, and the Kline the most sensitive of the three tests employed. In the estrinized animals, positive reactions appeared from 23 to 29 days after inoculation, and remained positive for from 54 to 114 days.

It is of interest that there was no important difference between the two groups in the duration of the phase of positive reaction. Because of the much longer period of latency among the treated rabbits, a reduction in the positive phase of the reactions might have been expected. In the rabbit, positive serological reactions are limited, as a rule, to the period of active lesions, particularly of the testis.

Body weight. The ability of an animal to maintain a normal body weight during the course of an infection is an expression of the efficiency of its resistance to the infection. Table 6 gives the mean weights of the animals in both groups. The figures at the beginning of the experiment are from weights taken the week before the administration of estrogenic substance began, and those at the end of the experiment were taken the week before death. The initial and terminal weights adequately indicate the behavior of the animals in this respect, as variations during the interim followed the same trend.

During the time the animals in the control group were under observation, each lost on the average of 142.8 ± 75.6 gm., but those treated with estrogenic substance showed no significant variation in weight. As treatment was the only variable condition affecting the two groups, it is assumed that the differences observed accrued from the beneficial effects of estrogenic treatment.

GENERAL DISCUSSION

The bodily changes induced by the action of the urinary extract demonstrated the potency of the preparation and its physiological relationship to the estrogenic

hormones already isolated in crystalline form. The induction by the extract of the typical phenomena of estrus in the vaginal mucosa of ovariectomized rats has already been discussed in Part I, as also has the stimulation of the growth of the mammary glands and the initiation of lactation. Other evidence of the feminizing influence of the preparation was shown by the involution of the penis to a form resembling the clitoris and the urogenital vestibulum of the female rabbit; by the recession of the scrotal sacs, the atrophy and partial ascent of the testes; and by the development of a dewlap.

The response of the estrinized animals to syphilitic infection is to be viewed against the background of their feminized condition. It is reasonable to assume, therefore, that changes induced by estrogens in tissues and organs reacting to infection, as for example the testis, should affect to some extent the pattern of the reaction, at least in those structures. However, when the disease is generally modified without relation to the tissues manifestly affected by the estrogens, it is necessary in attempting to explain the phenomenon to go beyond the local effects of treatment and seek an explanation which comprehends changes affecting the immunologic processes in general.

The effect of the estrogens was inhibitory to the progress and degree of reaction in the testis and scrotum. The duration of treatment, and presumably the concentration of estrogens in the body at the time of inoculation, bore a direct relationship to the character of the primary response to infection. This was shown by the differences between the two experiments in the course of the disease. In the first, where inoculation and the beginning of estrogenic treatment nearly coincided, primary orchitis appeared earlier, lasted longer, and was more severe than in the second experiment in which the animals were subjected to a much longer preparatory treatment before inoculation. In the first experiment, the primary reaction took place in testes which had not shown any change in size, and in animals that had developed none of the physiological responses to estrogenic therapy, if enlargement of nipples is excepted. It is also of interest that in this instance the maximum size of the testicular lesion was definitely greater among the treated animals. This fact, together with the quicker response to inoculation, suggests that the initial effect of the estrogenic substance was one which temporarily increased the susceptibility of the testis to the infection.

In the second experiment the feminizing influences of the estrogenic hormones were well established before the onset of orchitis. In this experiment, in which the animals showed considerable testicular atrophy at the time of inoculation, no reaction in the inoculated testis was detected in some of the rabbits. However, the scrotal tissues of the refractory animals in all but one instance did react to form a chancre, although these lesions did not extend to involve the testis.

In like manner, the metastatic testicular reaction did not develop; only one animal in 20 treated with estrogenic substance had an orchitis of the uninoculated testis. In this rabbit the orchitis was mild and transitory. Scrotal chancres, however, appeared earlier and were more destructive in the estrinized animals. Moreover, they were identified with the reaction at the site of inoculation and not with the metastatic reaction as in the normal rabbits.

The question arises as to the respective relationships of the testicular and scrotal elements of the reaction to the subsequent course of the disease. It is necessary, first, to consider the reactions in these structures together as constituting one major phase of the infection. This we shall designate the early, or testicular phase as distinguished from the late, or generalized phase. Upon the basis of this definition we have compared the incidence of both the testicular and generalized phases in the two groups. This showed that the former occurred with about equal frequency in treated and normal rabits, but that the generalized phase developed with much less frequency among the treated animals.

On the same basis, we have also compared the duration of the testicular phase in the two groups. This showed a highly significant reduction in the length of the combined reactions of testis and scrotum among the estrinized animals.

Looking next at the time relationships between inoculation and the early signs of disease, it is apparent that the initial reaction in the testis was retarded among the animals under estrogenic treatment, but with the succeeding stages of primary scrotal and metastatic testicular reactions, the estrinized animals showed a significant shortening of the respective incubation periods. With the more rapid healing of these lesions, the infection progressed with greater rapidity so that the testicular phase of the disease was confined to a much briefer period of time than in the normal animals.

When the testicular and scrotal components of the reaction are considered individually, the differences referred to above are found to result chiefly from the suppression of the testicular reaction. There was, however, an acceleration of the appearance of the primary chancre among the treated animals.

In summarizing the results thus far discussed, the mildness of the primary orchitis and the absence of metastatic orchitis were the most noteworthy features of the disease among the estrinized rabbits. There was also a disturbance of the normal sequence of events as the disease progressed, and besides, the duration of the entire early period of the infection was remarkably shortened. It was also apparent that the scrotal tissues were not affected by treatment to the same degree as were the tissues of the testis.

Progressing from the primary chancre to metastatic orchitis and chancre, the acceleration of response to infection, became gradually less until the reverse relationship in time came into play with the onset of generalized lesions. That is to say, there was retardation in the rate of appearance of the lesions in bone, skin, and eye. Yet it would seem that the brief testicular response conferred upon the treated animals a more perfect protection against the later manifestations of disease than did the more intense and extended testicular reactions developed by the normal animals.

Whether or not one chooses to place this emphasis upon the immunologic relationship between the two phases of the disease, it is at least apparent that the estrinized animals developed an increasing ability to withstand the injurious effects of the disease the longer they had been subjected to the influence of estrogenic substance. This was well shown by the fact that the treated animals were definitely more resistant to the development of generalized lesions than the normal animals. More than a third of their number failed to show generalized foci of infection, and those that did had fewer lesions to the animal. Thus it is apparent that throughout the entire course of the disease the feminized rabbits showed a more perfect defense against infection. This same advantage was also manifest in a more stable body weight and in the development of a more luxuriant and heavier coat of hair.

The mechanism by which resistance to syphilis was enhanced is not revealed through these experiments. Certain pertinent questions, however, suggest themselves, the answer to which might shed some light on the way in which estrogenic treatment affected the behavior of the animals toward infection. These questions relate for the most part to the site of action of the estrogens, and the probable relationship of the tissues affected to the localization and development of the processes of syphilitic disease in the rabbit. In this connection, we refer not so much to the structures primarily affected by the estrogens, such as the anterior pituitary gland, as to those tissues which were modified indirectly by their action.

That the virulence of the organisms was not impaired, at least permanently, by estrogenic action (a fact later demonstrated by the transfer of tissues from the estrinized animals to normal animals) is proof that the loss of susceptibility of certain structures, especially of the testes and to a less degree of the bone and skin, depended upon alterations of tissue tolerance rather than upon attenuation of the virulence of the organisms themselves.

An explanation based on the traditional conception of the nature of immunological responses necessarily assumes that the estrogens served to stimulate the body to an active defense against invasion by the treponemes. There remains, however, another possible mode of action which might explain, at least in part, the increased tolerance to infection. This takes account of the property of the estrogens to alter or destroy certain cellular structures, or those cellular attributes, which normally favor the growth and multiplication of spirochetes in the rabbit, and thus enhance their pathogenic proclivities in those tissues, and perhaps even in tissues and organs remote from the site of such cellular changes.

The latter possibility is suggested by the striking modifications of the disease which took place in the testes. These organs, as a rule, are highly vulnerable to syphilitic infection, and react with the formation of remarkable inflammatory lesions. After prolonged estrogenic action, however, there was a reversal of susceptibility, the testes becoming refractory to the disease. The increased adaptability to infection might have been due it seems to a selective action of the estrogens on certain cellular functions, the suppression of which removed a factor that normally increases local tissue susceptibility and enables the organisms of syphilis more easily to invade the body and there to establish manifest disease. The fact that testicular atrophy and increased tolerance to syphilis seemed to proceed concurrently, and to be in relative agreement, is one reason for reaching this conclusion. Although one can only speculate on the possibility, it seems not unlikely that the absence from the testes of this enhancing factor might also have modified the general pathogenicity of the organisms either by restricting their dissemination from the primary focus, or by allowing a more effective immunity to develop during the testicular phase of infection.

SUMMARY

The course of syphilitic infection in two groups of adult male rabbits, homogeneous as to breed, age, and nutrition, was studied. Before intratesticular inoculation with *Treponema pallidum*, one group of animals was subjected to prolonged treatment with an estrogenic substance prepared by extracting human pregnancy urine with butyl alcohol. The animals were injected daily with the ether soluble fraction of this extract dissolved in olive oil. The estrogenic content of the preparation was assayed by the use of the vaginal growth effect on ovariectomized albino rats. The physiological effects of the estrogenic substance were manifest in the growth of the nipples and the mammary glands together with the stimulation of lactation; the feminization of the external genitalia and atrophy of the testes; the development of dewlap, a secondary sexual characteristic of the female rabbit; and in certain changes of psychosexual behavior.

Among the estrinized rabbits, the early manifestations of syphilis were much milder and followed a shorter course than among the sexually intact rabbits. The estrinized animals showed an increasing ability to withstand the injurious effects of the disease the longer they were treated. This was shown by the greater frequency with which they failed to develop generalized foci of infection.

The resistance to disease developed by the testis was the most noteworthy modification of the reaction to infection, although the skeletal and cutaneous tissues behaved in a similar manner. The period of latency was greatly prolonged among the treated animals, and they remained in a better state of nutrition.

The possibility of testicular changes, induced by estrogenic action, being directly related to the inhibition of infection is discussed.

REFERENCES

1. Frazier, C. N., and C. K. Hu: *Endocrinology* 28: 283. 1941.
2. Nichols, H. J.: *J.A.M.A.* 63: 466. 1914.
3. Brown, W. H., and L. Pearce: *J.A.M.A.* 77: 1619. 1921.

STUDIES OF THE PROGESTERONE-LIKE ACTION OF DESOXYCORTICOSTERONE ACETATE IN WOMEN[1]

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RECENTLY several members of our group (1) reported that a male excreted sodium pregnandiol glucuronide in the urine during the course of therapy which involved intramuscularly administered desoxycorticosterone acetate in oil. In view of this observation and of reports that desoxycorticosterone acetate was capable of inducing progesterone-like responses in certain experimental animals (2), a study of the possible progestational action of desoxycorticosterone acetate in women was initiated.

METHODS

Two groups of women were studied. One group of 5 women (average age 25 years) was characterized by the common symptom of secondary amenorrhea, the duration of which had ranged from 3 to 9 months. The other group comprised 3 women (average age 29 years) who received desoxycorticosterone preoperatively.

The procedure in the first group was as follows. Endometrial responses prior to therapy were sampled by biopsies. Pretreatment levels of urinary excretion of 17-ketosteroids (androgenic metabolic products), quantitated by Oesting's method (3) after preliminary extraction by the method of Cuyler and Baptist (4), and of sodium pregnandiol glucuronide (metabolic product of progestin), determined by the method of Venning (5) were established. The urinary titrations were continued during therapy and endometrial studies were made upon biopsies taken at the onset of bleeding or at the conclusion of therapy. The treatment schedule employed was essentially the following. Estrone (10,000 I.U.) or estradiol benzoate (2000 R.U.) was given intramuscularly daily for 14 consecutive days; desoxycorticosterone acetate (5 mg.) and either estrone (10,000 I.U.) or estriol glucuronide (1800 D.U.) were given daily the next 14 days (therapy being discontinued, however, if bleeding began).

No endometrial responses were investigated in the 3 patients of the surgical group. Pretreatment and treatment levels of urinary excretion of 17-ketosteroids were determined in all 3 patients. In one patient the urinary excretion of sodium pregnandiol glucuronide also was studied. These patients received desoxycorticosterone acetate daily prior to surgery (5 to 20 mg.) and during the immediate postoperative period (5 to 10 mg.).

Received for publication July 29, 1940.
[1] A part of the expenses incurred in these studies was defrayed by funds available from grants to one of us (E.C.H.) by the Research Council of Duke University and by the Schering Corporation, Bloomfield, New Jersey.
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RESULTS

The clinical data secured during these studies are summarized in table 1.

Alterations in urinary titers of 17-ketosteroids. A total of 196 individual determinations was made. The average daily values for different patients prior to therapy ranged from 7 to 225 I.U. and during therapy from 10 to 219 I.U. The average daily value for the entire group was 63 I.U. prior to therapy and 57 I.U. during therapy.

Alterations in the urinary titers of sodium pregnandiol glucuronide. A total of 156 individual determinations was made. None of the patients excreted pregnandiol complex prior to therapy. During therapy a total of 18 mg. of sodium pregnandiol glucuronide was recovered during 3 of the 8 cycles investigated, an average recovery of 2.25 mg. per cycle.

Alterations in endometrial responses. All 5 patients studied had estrogenic endometria prior to therapy (3 hypoestrogenic and 2 persistent estrogenic). All 5 patients had estrogenic endometria at the conclusion of the various cycles of therapy (persistent estrogenic 6 and hyperestrogenic 1). Bleeding followed therapy in 4 of the 8 cycles studied.

TABLE 1. SUMMARY OF CLINICAL DATA ON PATIENTS TREATED WITH DESOXYCORTICOSTERONE ACETATE

Case No.	Age of patient	Before therapy: 17-Ketosteroids, daily average	Before therapy: Pregnandiol complex, total excretion	Before therapy: Endometrial response	Treatment formula	During therapy: 17-Ketosteroids daily average	During therapy: Pregnandiol complex, total excretion	Endometrial response at end of treatment
		I.U.	mg.			I.U.	mg.	
D_1	31	41	0	E−	E→Es+Dca(60)	30	0	E
D_2	23	74	0	E	Dca(20)	—	—	E+
		—	—	E+	EdB+E→Es+Dca(25)	52	0	E
		51	0	E	EdB→E+Dca(45)	35	6	Eo
D_3	22	16	0	E−	E→Es+Dca(35)	24	3	E
		7	0	E	E→E+Dca(50)	10	0	Eo
D_4	22	35	0	E	E→E+Dca(45)	16	9	0
D_5	27	45	0	E−	E→E+Dca(50)	76	0	Eo
D_6	27	225	—	—	Dca(130)	219	—	—
D_7	25	60	—	—	Dca(85)	19	—	—
D_8	35	78	0	—	Dca(25)	84	0	—

Code to Abbreviations:

Endometrial responses, —o no bleeding occurred
E− hypoestrogenic
E persistent estrogenic
E+ hyperestrogenic

Therapy,— E estrone
Es estriol glucuronide
Dca desoxycorticosterone acetate
EdB estradiol benzoate
The numerals in parentheses indicate dosage in mg. of desoxycorticosterone acetate.

Untoward clinical responses. These were minimal. Two patients experienced definite disturbances in water balance, which involved decreased urinary volume, generalized edema, sensation of thirst, slight acceleration of pulse and slight elevation in blood pressure.

DISCUSSION

No evidence was encountered that desoxycorticosterone acetate is altered during metabolism following injection into androgenic substances. The androgenic properties demonstrated in the intact adrenals of the immature rat (6) and of aqueous extracts of adrenals (7) are not due doubtlessly to desoxycorticosterone acetate. An androgenic property for desoxycorticosterone acetate has been affirmed (8) and denied (9).

The fact that injections of desoxycorticosterone acetate are followed by the urinary excretion of significant amounts of sodium pregnandiol glucuronide in the instance of a male studied, but are not associated with any clear-cut augmentation of similar urinary titers of the 5 females studied, is difficult to explain. It may indicate a sexual difference in the manner of metabolizing the sterol.

The studies on the endometriotropic effects of desoxycorticosterone acetate are conclusive. No progestational activity was demonstrated despite the fact that an average of 41.2 mg. of desoxycorticosterone acetate was given per cycle of therapy and despite the fact that 1 patient was given 3 consecutive cycles of therapy. The requirement of estrogenic priming of the endometrium prior to expected progestational responses was fulfilled.

It would be desirable to study the effects of desoxycorticosterone acetate upon estrogenic metabolism since Salmon (10) has observed estrogenic effects on the vaginal mucosa of postmenopausal women during therapy with desoxycorticosterone acetate. It may be that some or even all of the desoxycorticosterone acetate injected is transformed into estrogens. These studies do not permit any statements in this regard.

SUMMARY

The metabolism of and the endometriotropic responses to injected desoxycorticosterone acetate were studied in 8 women. This therapy produced no significant alterations in the urinary excretion of 17-ketosteroids, determined by Oesting's method or of sodium pregnandiol glucuronide, determined by Venning's method. Despite pretreatment priming of endometria with estrogens, no progestational changes were produced in the endometria of the 5 patients investigated. One patient received 3 cycles of therapy. The average amount of desoxycorticosterone acetate given was 41.2 mg./cycle when endometriotropic responses were investigated.

We are indebted to the following pharmaceutical organizations for liberal supplies of the sterols used: estrone furnished by Parke, Davis & Company (theelin), and E. R. Squibb & Sons (amniotin); estriol glucuronide furnished by Ayerst, McKenna & Harrison (emmenin); desoxycorticosterone acetate furnished by Schering Corporation (cortate); estradiol benzoate furnished by Schering Corporation (progynon-B) and Ciba Pharmaceutical Products Incorporated (ben-ovocylin).

REFERENCES

1. Cuyler, W. K., C. Ashley and E. C. Hamblen: *Endocrinology* (in press).
2. Hoffman, A.: *Zentralbl. f. Gynäk.*, 62: 2694. 1938; Miescher, K., W. H. Fischer and E. Tschopp: *Nature* 142: 435. 1938; van Heuverswyn, J., V. J. Collins, W. L. Williams and W. A. Gardner: *Proc. Soc. Exper. Biol. & Med.* 41: 552. 1939; Wells, J. A., and R. R. Green: *Proc. Am. Physiol. Soc.*, p. 652. 1939; Robson, J. M.: *Proc. Physiol. Soc.*, p. 21. 1939; Hohlweg, W.: *Zentralbl. f. Gynäk.* 63: 1143. 1939; Leathem, J. H., and R. C. Crafts: *Endocrinology* 26: 731. 1940.
3. Oesting, R. B.: *Proc. Soc. Exper. Biol. & Med.* 36: 524. 1937; 39: 76. 1938.
4. Cuyler, W. K., and M. Baptist: *J. Lab. & Clin. Med.* (in press).
5. Venning, E. H.: *J. Biol. Chem.* 126: 595. 1938.
6. Burrill, M. W., and R. R. Greene: *Proc. Soc. Exper. Biol. & Med.* 40: 327. 1939; *Endocrinology* 26: 645. 1940; Howard, E.: *Proc. Am. J. Physiol.*, p. 539. 1939.
7. Hodler, D.: *Compt. Rend. Soc. de Biol.* 122: 512. 1936.
8. Greene, R. R., and M. W. Burrill: *Proc. Soc. Exper. Biol. & Med.* 43: 382. 1940.
9. Hooker, C. W., and V. J. Collins: *Endocrinology* 26: 269. 1940.
10. Salmon, W. J.: *Proc. Soc. Exper. Biol. & Med.* 41: 515. 1939.

THE REGULATION OF CORPUS LUTEUM FUNCTION BY HYPOPHYSIAL LUTEOTROPHIN[1]

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Since the original discovery of the fact that gonadal development is influenced by two hypophysial hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (1, 2, 3), ample evidence has been accumulated to show that these two substances are separate and distinct (4–7). The periodic secretion of these two substances by the pituitary is modified in part by the ovarian hormones, and on the basis of these pituitary-ovarian interactions various theories concerning the control of the estrous cycle have been advanced. A number of questions concerning these interactions remain unanswered, and in particular no satisfactory mechanism has been suggested for the control of the luteal phase of the cycle or pseudopregnancy. The recent finding that progesterone effectively blocks the release of luteinizing hormone from the pituitary (8, 9, 10), taken together with certain other considerations, led to the suggestion (10) that while LH is responsible for the conversion of follicles into corpora lutea, a third hypophysial gonadotropic factor is concerned with luteal function.

It has previously been shown (11) that the placenta of the rat contains a hormone which will maintain the function of formed corpora lutea in hypophysectomized rats. This action was attributed to a species-specific hormone of pregnancy, called a cyonin, the function of which is to prolong luteal function during gestation (12). Evidence was given to show that rat cyonin differs from the known hypophysial hormones and from the cyonins of the human being and of the mare. These reports left unanswered the question of luteal activity in the non-pregnant animal.

Evidence has been presented to indicate that the hypophysial factor responsible for luteal function is not one of the known gonadotropins. Dresel (13), and Lahr and Riddle (14) have shown that purified lactogenic hormone preparations would inhibit the estrous cycle in normal adult mice and rats and found that the ovaries of such animals contained a number of large corpora lutea. On the other hand Nathanson and Fevold (15), while confirming this observation, demonstrated that luteinizing hormone extracts containing no lactogenic activity would accomplish a similar result. Lyons (16), using purified fractions obtained by a different chemical procedure found that the daily injection of 1 mg. of an extract of either lactogenic or adrenotropic hormone would stop the estrous cycle in adult rats and result in the persistence of one crop of active corpora lutea. The possibility that the estrous cycle may be disturbed by one of several different mechanisms indicates that the intact animal is poorly suited for tests of corpus luteum stimulation. The above observations have been substantiated, however, by the work of Evans, Simpson, and Turpeinen (17), who demonstrated that deciduoma formation could be induced in adult hypophysectomized rats treated with either lactogenic or adrenotropic hormone preparations.

Some hypophysial principle therefore seems to be responsible for the functioning

[1] Aided by a grant from the Rockefeller Foundation Fluid Research Fund of Johns Hopkins University.

of formed corpora lutea, and the experiments to be reported here were undertaken to define more clearly the chemical and biological properties of this hormone.

In speaking of a substance which will maintain the functioning of formed corpora lutea it has been found convenient to use the term "luteotrophic" substance or "luteotrophin."

This investigation necessitated, in the first place, the development of a satisfactory method of testing for a luteotrophic substance. The test previously used to detect a corpus luteum-stimulating substance in rat placentae depended upon the decidual reaction in hypophysectomized pseudopregnant rats (11). This test has been found to be satisfactory also for the assay of hypophysial luteotrophin but theoretically it is open to the objection that deciduoma formation is easily inhibited by excess estrogen (18, 19). A pituitary extract which stimulates the secretion of ovarian hormones in an inappropriate ratio might conceivably fail to support decidual growth, and the presence of luteotrophin in such an extract would thereby be obscured. To avoid this difficulty, advantage has been taken of the inhibitory influence of active corpora lutea on the cornifying effect of estrogen on the vagina.

The second requirement was to prepare hypophysial extracts which would contain the luteotrophic agent, and by the use of various procedures to differentiate this factor from or to identify it with the recognized pituitary hormones. To this end various pituitary fractions have been tested for luteotrophic activity by a method which is described below.

EXPERIMENTAL

Pituitary extracts. Five batches of fresh frozen pituitary glands of sheep in 500 to 1,000 gm. lots were finely ground by passing the frozen tissue three times through an electric mill. Crude gonadotropic extracts were made by the methods of Fevold (4) and of Chow, Greep and Van Dyke (5) and then fractionated by the method of Fevold, Lee, Hisaw and Cohn (20) into follicle stimulating and luteinizing hormones. As either of these methods effectively extracts the gonadotropic and thyrotropic hormones the residual tissue after such treatment was further extracted in a somewhat similar manner to remove all traces of these hormones. To the residue from 1 kg. of pituitaries 10 volumes of water was added and the pH adjusted to 8.0 with 1.0 N NaOH. After thorough stirring the suspension was freed of tissue particles by passing through a graded series of sieves and was then centrifuged at 2,000 R.P.M. for ½ to 1 hour. The turbid supernatant fluid was removed and acidified with 1.0 N acetic acid. The copious precipitate which formed was collected by filtration, resuspended in water and treated again in the above manner. This procedure was repeated 3 or 4 times. In the several batches of pituitary glands treated in this way the pH at which the large isoelectric precipitate was collected varied from 4.8 to 5.4 but in each case an abundant precipitate was obtained which left the supernatant clear. By this method extracts were obtained free of FSH, LH and thyrotropin. These extracts, referred to as "crude luteotrophin," were contaminated with considerable amounts of growth and adrenotropic factors.

Four lots of sheep pituitaries of 500 gm. each were treated by the method of Lyons (16). This method yields a purified product containing the adrenotropic and lactogenic hormones and recently has been applied by Li, Lyons and Evans (21) to obtain the lactogenic factor in chemically pure form. The residual pituitary tissue and each of the discarded fractions were dried with acetone and saved for testing.

Three small batches of finely ground fresh sheep pituitary tissue were dried with acetone and powdered. This material was injected as a suspension in 0.9% sodium chloride solution. In each case the dosages were expressed as equivalent weights of fresh glandular tissue. In some cases the progress of purification was followed by determinations of total nitrogen by the micro Kjeldahl method.

Assay method. Corpora lutea were induced in the ovaries of young (23 to 30-day-old) female rats by the daily injection of 2 I.U. of chorionic gonadotropin for 4 days. On the fourth day of treatment, when vaginal estrus had appeared, an injection of 20 micrograms of estradiol dipropionate was given. A continued supply of estrogen was then maintained by

further injections of 10 γ twice weekly, a dosage that was sufficient to maintain continuous vaginal cornification in oöphorectomized rats. After one to three days of vaginal cornification resulting from this combined treatment, the vaginal smears were of the diestrous type because of the vaginal mucification induced by the active corpora lutea. Hypophysectomy was performed on the first or second day of diestrus, 7 to 8 days after the first injection of gonadotropin; the administration of pituitary extracts was begun immediately and continued twice daily for periods of four to fourteen days. A recurrence of vaginal cornification after hypophysectomy in animals thus prepared would indicate cessation of corpus luteum function, while sustained vaginal mucification would be expected if the function of the corpora lutea was maintained. It was thus possible to determine by the fourth day following hypophysectomy whether or not a given dosage of luteotrophin was active.

The animals were weighed daily and at autopsy the weights of the adrenals, thyroids, gonads and sex accessories were taken; the ovaries and vaginae were prepared for microscopic examination, and the mammary glands examined as whole mounts. The data which follow comprise 206 hypophysectomized animals prepared in the above manner. The completeness of the operation was confirmed by examination of the hypophysial region under a dissecting microscope.

The use of such animals for the assay of luteotrophin is based upon the following considerations. A group of 24 animals were treated in the above manner with chorionic gonadotropin for 4 days followed by estradiol dipropionate. After 1 to 3 days of vaginal cornification diestrous smears appeared and persisted for 13 to 29 days (average 18 days) before cornification returned. This period of diestrous smears from mucified vaginal mucosae in the presence of injected estrogen lent confirmation to the recognized fact that artificially induced corpora lutea in immature rats are active. The duration of this corpus luteum activity agrees well with the observations of Wolfe (22), and others (23–27). That this function is mediated by the intact hypophysis is shown by the following two groups of animals similarly prepared. The first group of 10 animals was oöphorectomized on the second day of diestrus following the combined treatment of gonadotropin and estrogen, while the second group of 20 animals was hypophysectomized. In both groups vaginal smears occurred within 72 hours of operation, and complete cornification within 96 hours. Thus, as far as could be determined by daily vaginal smears, hypophysectomy terminated luteal function as effectively and just as quickly as removal of the ovaries.

In order to establish that artificially induced corpora lutea in immature hypophysectomized rats may be made to function injections of crude extracts of rat placentae were begun immediately after hypophysectomy. Of 10 animals thus treated all showed continued diestrous vaginal smears; they were killed at intervals from 3 to 14 days following operation and showed a well-maintained mucification of the vaginae, lack of estrous distention of the uterus, and lobular development of the mammary glands. The ovaries decreased in weight because of a disappearance of large follicles, while the cells of the corpora lutea were enlarged. Six animals were treated in the same way following the removal of both ovaries at the time of hypophysectomy. Estrous smears appeared on the fourth day and continued during 10 days of treatment. These findings substantiated the earlier observation that corpus luteum activity follows the injection of rat placental extracts in hypophysectomized pseudopregnant animals (11). How long this artificial activation can be maintained in the absence of the hypophysis has not yet been determined because of the limited supply of rat placentae.

RESULTS

The method of assay used is shown in figure 1, which depicts diagrammatically the sequence of vaginal smear changes in several illustrative experiments. On the left in each case is the temporary period of vaginal cornification induced by the gonado-

tropic agent used in forming the corpora lutea. Following this is a period of diestrus signaling corpus luteum activity; early in this phase the hypophyses were removed. The vaginal changes which followed hypophysectomy are shown on the right. The time sequence varied somewhat in the different groups and figure 1 comprises an average of the behavior of individual animals. At the top of the figure is shown the response of the two groups of hypophysectomized animals already referred to.

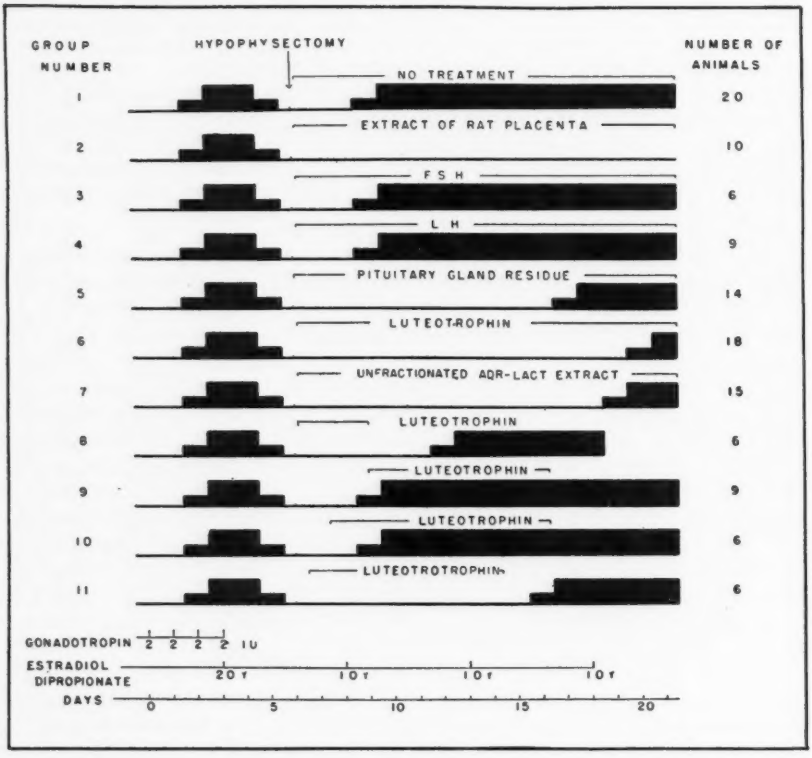

Fig. 1. Diagrammatic representation of vaginal smear changes in 11 groups of rats treated with various preparations. The solid black areas represent complete vaginal cornification, the intervening base line indicating diestrous smears. On the left are shown the brief periods of cornification resulting from the treatment with gonadotropin and estrogen in the amounts given at the bottom of the figure. The time of hypophysectomy, as shown, follows the return of diestrous smears. The long horizontal brackets enclose the period of postoperative treatment with the several types of extract.

Group I, representing animals without treatment showed permanent vaginal cornification beginning 4 days after hypophysectomy, while the injection of rat placental extract brought about sustained diestrus in *Group II*. *Groups III* and *IV* were treated with follicle-stimulating hormone and luteinizing hormone respectively. These extracts were prepared by the method of Fevold in one instance and by that of Chow, Greep, and Van Dyke in a second. Another series of 5 rats were treated with a purified preparation of LH kindly supplied by Dr. Fevold. In no instance was there any indication that FSH or LH sustained luteal function. In 6 groups totalling 22 animals the daily dosage of FSH was varied from 1 to 25 U and that of LH, from 1 to 20 U

(0.2 to 5.0 gm. equivalents of fresh sheep pituitary gland). One unit of each preparation was the amount required to double the weight of the ovaries (FSH) or seminal vesicles (LH) in normal immature rats as defined by Fevold (4, 28). These results confirm the findings of Greep (29) who showed that treatment with FSH or LH was ineffective in inducing luteal function in hypophysectomized adult rats. Animals of *Group V* in figure 1 were treated with the pituitary residue remaining after the removal of the gonadotropin fraction by the methods mentioned above. Sustained luteal activity occurred in these animals with a daily dose as low as 0.1 gm. equivalent of fresh whole sheep pituitary gland.

The repeated treatment of this residue by the method given above resulted in no detectable loss of luteotrophic activity as approximated by the method described. The resulting luteotrophic fractions were consistently active in daily doses of 0.1 gm. equivalent of fresh pituitary gland (*Groups V* and *VI*, fig. 1). This treatment removed all detectable FSH, LH and thyrotropin as shown by tests on hypophysectomized male and female immature rats. A total dose of 5 gm. equivalent of fresh pituitary (50 times the minimal effective dose for luteotrophic activity) injected over 4 days failed to produce a significant increase in the weights of the ovaries, uteri, testes, seminal vesicles, prostates or thyroids of the test animals. However, adrenal growth was produced at all dosage levels adequate for a luteotrophic effect and dosages 0.2 to 1.0 gm. equivalent produced an increase in body weight. It was apparent that this method of extraction effected little separation of the desired substance from adrenotropin and the growth factor.

The fractions obtained by the method of Lyons were tested for luteotrophin as well as for FSH, LH, thyrotropin, adrenotropin and growth hormone. The lactogenic-adrenotropic fractions obtained by this method exhibited luteotrophic activity in the daily dose of 0.5 gm. equivalent of fresh gland (*Group VII*, fig. 1). The most active preparation obtained by this method required a daily dose containing 17γ of total nitrogen to sustain luteal function. This dosage was equivalent to 0.6 gm. of fresh pituitary gland. Doses of 50 times the minimal effective dose for luteotrophic activity did not stimulate growth and contained no detectable thyrotropin, FSH or LH.

The fractions which are discarded in the extraction method of Lyons were tested in a similar way. Both the glandular residue after two extractions and the abundant ammoniacal acetone precipitate after resolution and re-precipitation exhibited luteotrophic activity when tested at dosage levels of 1.0 and 0.5 gm. equivalents respectively of fresh gland but these two residues contained only small amounts of adrenotropin.

When acetone dried sheep pituitary powder was tested the results were less clear and it was difficult to establish a minimal effective dose. With daily dosages of 0.01 gm. (equivalent to 0.07–0.1 gm. fresh pituitary tissue) vaginal mucification was well maintained but the development of successive sets of corpora lutea made conclusions difficult as to the functional state of any one group of corpora lutea. With smaller dosages the vaginal smear tended to show a cycle and a definite endpoint was not obtained.

With all extracts the minimal effective dose was equivalent to a fairly large amount of pituitary tissue. This approximated 0.1 gm. equivalent daily for the acetone dried powder, for the residue remaining after extracting the gonadotropin and thyrotropin and for the crude luteotrophin. The lactogenic-adrenotropic fractions were consistently active in dosages of 0.5 gm. equivalent. Thus only one fifth of the activity present in the original tissue was recovered and this may be accounted for by the failure to remove all activity from the discarded residues.

Influence of delayed treatment. The persistence of corpora lutea of apparently normal histologic appearance after hypophysectomy raised the question of their functional capacity under the influence of luteotrophin after various periods of hypophysial deprivation. Consequently in 31 animals various periods of time were allowed to elapse after hypophysectomy before treatment with luteotrophin was begun. In 9 animals 72 hours were allowed to elapse; by this time the vaginal smears were proestrous and the injection of luteotrophin for the next 5 to 8 days did not modify the appearance and persistence of continuous vaginal cornification. In 4 animals a 48-hour delay and in 6 a 30-hour delay resulted in a complete lack of effectiveness of subsequently injected luteotrophin. Of 8 animals in which a delay of 6 to 20 hours was allowed 7 showed activation while in 6 others in which treatment was begun 6 hours after hypophysectomy all were successfully maintained. These results indicate that following hypophysectomy a period of 20 to 30 hours is sufficient to allow for depletion of luteotrophin and that the disappearance of this substance is followed by an irreversible change in the persisting corpora lutea which renders them unresponsive to stimulation by luteotrophin. In 6 animals injections of luteotrophin were begun immediately after hypophysectomy and continued for 3 days only. Completely cornified smears appeared within 96 hours of the last injection indicating that corpus luteum function ceases after discontinuation of luteotrophin just as quickly as after hypophysectomy in the untreated animals. Consideration is given to the fact that 72 to 96 hours are required for the mucified vagina to become fully cornified after the influence of the corpus luteum hormone has been removed. It appears therefore that the continuous and steady action of luteotrophin is an important factor in the maintenance of luteal function. This circumstance probably limits the accuracy of this type of assay method as a quantitative procedure.

Continued treatment with various preparations containing luteotrophin usually failed to maintain vaginal mucification beyond 10 to 13 days following hypophysectomy. In three instances diestrous smears continued for 15 days but in all cases cornification returned in spite of continued injections. The cause for this limited span of function has not been determined. It is possible that the degeneration of the corpora lutea may be a consequence of the repeated periods of luteotrophin deprivation which occur between successive injections and that their life span would be lengthened by a continuous action of the hormone. A second factor may be that the dosage of luteotrophin was too large or too small for maximal duration of function. An alternative explanation may have as its basis the inherent maximal life span of corpora lutea under the stimulus of luteotrophin. Such a view receives support from the observation that the usual period of 10 to 13 days of induced function corresponds to the usual length of pseudopregnancy; in pregnancy the added 10 days of luteal function is mediated by a different hormone of chorionic origin.

Influence of estrogen. As estrogen has been shown to influence the corpora lutea of the hypophysectomized rabbit (30, 31) and to enlarge the ovaries of the hypophysectomized immature rat (32, 33) two experiments were performed to determine whether or not estrogen influences corpus luteum function in the rat. Five animals, prepared as described above, were injected daily with 200 γ of estradiol dipropionate, beginning immediately after hypophysectomy. None of these animals exhibited any indication of luteal function. A possible synergistic effect of estrogen on luteotrophin was sought in another group of 6 animals to which no estrogen was given. Luteotrophin induced in these animals the usual changes in the ovaries and vagina with the exception that the height of the mucified vaginal mucosa was somewhat less than when estrogen was given as well.

Influence of LH, thyroid and adrenal cortex. The demonstration by Greep (28)

that LH and mixtures of FSH and LH promptly destroy the corpora lutea of hypophysectomized rats suggested the following experiment. A group of 6 animals prepared in the above manner was treated with an effective dose of luteotrophin for 10 days. On the third day of this treatment, 20 U. of LH were also given daily for 4 days. There was no indication from the vaginal smear that corpus luteum function was thereby impaired and at post mortem examination on the eleventh day the corpora lutea were not decreased in size. Therefore the destructive action of LH seems to affect only those corpora lutea which are not maintained in a functional state by luteotrophin.

Attempts to show that the action of luteotrophin is mediated by some other endocrine gland have thus far proved unsuccessful. In a group of 4 rats the thyroid and adrenals were removed at the time of hypophysectomy. Luteotrophin treatment in these animals effectively sustained luteal function.

Ovarian effects. Histological preparations of the ovaries and vaginae were made by the usual paraffin technic and stained by the hematoxylin-eosin and by Mallory's azan methods. The vaginal sections served to confirm the vaginal smears, mucification being prominent during the periods of active luteal function when the smears were of the diestrous type. The histological appearance of the ovaries varied with the type of extract given and with the duration of treatment. At the time of hypophysectomy one crop of well-formed corpora lutea were found in ovaries which contained follicles of various sizes and healthy interstitial tissue. Following hypophysectomy in animals which received no further treatment the follicles were found to be largely degenerated. The most mature healthy follicles contained but a small antrum, the interstitial tissue was atrophic but the corpora lutea persisted during the 14 days following hypophysectomy and underwent little change detectable by either staining method. There was a slight decrease in the size of the corpora lutea owing to a reduced size of the corpus luteum cells. These changes have been fully described by Smith (34).

Crude luteotrophin and the lactogenic-adrenotropic fraction of Lyons caused a pronounced effect upon the cells of the corpora lutea. There was a marked increase in cytoplasm which stained intensely with the blue of Mallory's stain and which had a finely granular appearance. The size of the cells was increased and the corpora lutea were thereby enlarged. These changes were most marked 5 to 7 days after hypophysectomy while signs of degeneration became conspicuous with prolonged treatment. Ten to 12 days after hypophysectomy the cytoplasm of the cells was found to be vacuolated, the spaces between the cells previously inconspicuous were open and the blood sinuses often contained blood. These signs of regression were noticeable only when the vaginal smear showed that the corpora lutea were no longer functional. It appeared that cessation of function as shown by the vaginal smear antedated signs of regression in the corpora lutea by 2 to 4 days. The parallelism between these histological changes and evidence of corpus luteum function under the influence of the various pituitary fractions indicated that the two effects were mediated by a common substance.

Mammary lobule proliferation. The mammary glands of the hypophysectomized rats used in these experiments were prepared in whole mounts. Glands from 135 animals treated in various ways were available for study. Following the combined treatment with gonadotropin and estrogen a considerable extention of the duct tree occurred and by the 7th or 8th day at the time when the animals were hypophysectomized a proliferation of end buds had resulted in early lobule formation similar to that normally seen on the 6th to 8th day of pseudopregnancy or pregnancy. Following hypophysectomy and without further treatment there was a prompt regres-

sion of the mammary glands within 4 to 6 days. They were composed of extended but bare ducts with but few finer twigs. As mentioned above the glands of hypophysectomized rats treated with crude rat placental extracts continued to proliferate and presented after ten days of treatment a picture resembling that seen on the 12th to 16th day of normal pregnancy. Whole pituitary powder produced a similar but more marked effect and this extensive lobule proliferation was also noted after treatment with the crude luteotrophic fraction. With various preparations of crude luteotrophin the degree of lobule proliferation varied markedly. With some the mammary gland was extremely proliferated and resembled in structure that seen at term in normal pregnant rats the total size of the gland remaining small. In some instances considerable secretion occurred resulting in pictures resembling early lactation. The mammary glands of animals treated with the lactogenic-adrenotropin fraction prepared by Lyons' method showed little or no proliferation. With some extracts the end buds showed some activity but in most instances the regression after hypophysectomy was not prevented although the activity of the corpora lutea was maintained.

It was thus apparent that there was no parallelism between the proliferation of lobules in the mammary gland and the maintainance of corpus luteum activity in hypophysectomized rats. Of those extracts which maintained corpus luteum function some induced mammary changes resembling pregnancy while others had no such effect and still others did not even prevent the regression which follows hypophysectomy. Mammary changes of the type described above were first experimentally induced by Corner (35) in intact rabbits and recently evidence has been reviewed by Gomez and Turner (36), and Lewis and Turner (37) leading to the suggestion that a special hypophysial factor is concerned called mammogenic hormone II.

DISCUSSION

Evidence for the essential role of the pituitary in luteal function in rats was given by Pencharz and Long (38) and Selye, Collip and Thomson (39) who demonstrated that pregnancy was promptly terminated by hypophysectomy performed prior to the tenth or eleventh day. Hypophysectomy later in pregnancy did not disturb gestation and it has since been demonstrated that the continued function of the corpora lutea during the second half of gestation in the rat is brought about by a corpus luteum stimulating substance from the placenta.

Greep (29) showed that the persisting corpora lutea of hypophysectomized rats are non-functional and that neither FSH nor LH is capable of activating such corpora lutea. This observation is confirmed by the experiments above. The latter hormone was found by Greep to exert a marked destructive action on such persisting corpora and a still more rapid regression resulted from a combined treatment with these gonadotropic pituitary fractions. These results were obtained on animals which had survived hypophysectomy for 15 days prior to treatment but good evidence was given to show that fresh corpora lutea responded to these hormones in a similar manner. Neither extracts of urine from pregnant women (29) nor preparations from the serum of pregnant mares (40) gave evidence of inducing luteal function in hypophysectomized rats.

That sustained luteal function may follow the injection of pituitary extracts in normal rats has been shown by several investigators. Teel (41) induced deciduomata in immature female rats treated with crude alkaline hypophysial extracts and this effect has been confirmed by Brouha (42) and others.

These results, however, do not prove that the corpora lutea induced by the treat-

ment are stimulated to activity by the extract or whether their function is induced by the animals' intact hypophyses. The latter view is sustained by the work of Cole (43) who has shown that ovulation can be induced in immature rats by a single injection of pregnant mare serum and that the resulting corpora lutea are capable of sustaining pregnancy without further treatmeent. The fact that the intact hypophysis of the test animal participates may probably explain the many observations indicating that deleterious influences such as the injection of toxic materials, exposure to cold or inadequate diet will modify the estrous cycle and induce pseudopregnancy or a state of anestrus.

It is apparent that corpus luteum function is a phenomenon entirely different from follicle stimulation or luteinization. The results reported above demonstrate that a separate hypophysial hormone, luteotrophin, is responsible for this phase of ovarian activity. It has been shown that luteotrophin is closely allied in its physical characteristics with the lactogenic and adrenotropic hormones. It is easily separated on the basis of solubility from FSH, LH and thyrotropin, and fairly readily dissociated from the growth factor. From the data obtained above, it appears to be distinct from adrenotropin, but it has not been established with certainty that adrenotropin can be prepared free from luteotrophic activity. Whether or not it is identical with the lactogenic hormone is a question as yet unanswered. The luteal function regularly accompanying lactation in such rodents as rats and mice may be taken as evidence that luteotrophin and the lactogenic hormone are related, but, in the majority of mammals lactation is unaccompanied by luteal function and in many the ovarian status during lactation is one of regression. In the human being normal menstrual cycles with ovulation followed by normal luteal phases may occur during lactation. If lactation and luteal function are mediated by a common substance this phenomenon is not easily explained. Furthermore, in most mammals luteal function during the estrous cycle, pseudopregnancy, and early pregnancy is as a rule unaccompanied by lactation. Mammary development in the direction of lobule formation pregresses during these periods, but the role of the lactogenic hormone in this mammary change is still uncertain and evidence given above indicates that luteotrophin is not responsible for this mammary effect in hypophysectomized rats.

Regulation of the estrous cycle. The introduction of a third factor in the interrelationship of the ovary and pituitary requires that the current concepts regarding the controlling mechanism of the reproductive cycle be modified. Sufficient evidence has accumulated on the common laboratory animals and the human being to warrant a tentative working hypothesis of the reciprocal interaction of these two endocrine glands. The following is suggested as one explanation of the observed facts.

As FSH is essential to follicular development beyond the stage of antrum formation, this hormone is presumably being secreted from the pituitary during the latter part of the luteal phase and during the follicular phase up to the time of ovulation. There is no conclusive proof that secretion of FSH is not fairly continuous during the non-pregnant cycle. The finding (5, 6, 44) that LH is essential to estrogen secretion from stimulated follicles may indicate that LH is secreted from the pituitary during the follicular phase of the cycle in amounts too small for luteinization. As the follicular phase advances, more and more LH may be produced and account for the increased output of estrogen from the ovary. Finally LH secretion or else the LH to FSH ratio reaches a sufficiently high level to bring about ovulation of those few follicles which, by virtue of their maturity, are most sensitive to luteinization, and atresia of other elements of an improper degree of development. Several lines of evidence (45, 46) indicate that corpus luteum hormone secretion begins when ovarian

follicles are undergoing preovulatory swelling under the influence of LH. Shortly after ovulation LH production is terminated by the action of progesterone upon the pituitary (10). As luteotrophin is essential to the secretion of corpus luteum hormone this substance is probably being released from the pituitary during the latter part of the follicular phase and throughout the whole of the luteal phase. The end of the luteal phase may be determined by an exhaustion of hypophysial luteotrophin. There is ample evidence (22–27) to show that estrogen brings about a luteotrophic effect in the presence of the hypophysis and it is probable that the stimulus of estrogen during the follicular phase conditions the pituitary to produce luteotrophin; this production then continues until this conditioning influence fails. An equally plausible explanation for the end of the luteal phase is that the corpus luteum has an inherent life span under the stimulus of luteotrophin and that the prolonged luteal function of pregnancy requires the further stimulus of a cyonin. There is no evidence available as yet to show that the secretion of luteotrophin is periodic.

The length of the period between the end of the luteal phase and the subsequent ovulation may be determined partly by the length of time required for a new set of follicles to reach maturity after the wide-spread destruction at the last ovulation, but it appears more probable that this period is determined by the time required for the pituitary to regain its ability to secrete LH in a quantity sufficient for ovulation after it has been under the repressive influence of corpus luteum hormone.

The repression of LH release by corpus luteum hormone accounts for the absence of ovulation or accessory luteinization while an active corpus luteum is present, and some evidence (47) points to the possibility that this repression is important to the subsequent release of sufficient LH to bring about the next ovulation. The absence of ovulation in pathological states of constant estrus may be explained on this basis. Estrogen is known to depress to a limited degree FSH production (48), and this mechanism is probably sufficient to account for the increased number of large follicles in the remaining ovary after unilateral oöphorectomy and to explain the limited but constant number of follicles maturing at a given time.

Certain species require, in addition to the above processes, a nervous stimulus in order to complete the cycle. The added stimulus of mating is essential in such animals as the cat, ferret, and rabbit in order that sufficient LH be produced to cause ovulation and in such animals as the rat, mouse, and hamster in order that the proper stimulus to luteotrophin production be supplied. In both these types the essential stimulus need be only short lived for severance of nervous connections to the pituitary after the stimulus does not interrupt the remainder of the cycle (49–54). It has recently been shown that estrogen may influence the ovary by a means other than via the hypophysis. Whether the action of estrogen on the corpus luteum of hypophysectomized rabbits and on the infantile ovaries of hypophysectomized rats is important to the control of the cycle is not readily apparent.

Both ovary and pituitary exhibit an inherent rhythm and a tendency to cyclic activity. The endocrine interactions between the two need only be regulatory in character in order that the reproductive cycle be controlled. Thus the action of ovarian hormones upon the secretion of the three gonad stimulating factors need only slightly stimulate or repress processes which already have a strong tendency to occur. At the present, therefore, the main regulatory action of the ovary on hypophysial activity appears to be the suppression of LH by corpus luteum hormone. Secondary in character is the action of estrogen in depressing the output of FSH and in conditioning the production of luteotrophin. The relative importance of these factors undoubtedly varies in different species of mammals.

SUMMARY

A method has been developed for the assay of a substance, hypophysial luteotrophin, which maintains the function of formed corpora lutea. Immature female rats were treated for four days with gonadotropin to induce corpus luteum formation and then treated with estradiol dipropionate to sustain a continued estrogen action. The modifying influence of the active corpora lutea on the estrogen action was used as an index of luteal function. Hypophysectomy in such animals was followed by all signs of unopposed estrogen action unless luteotrophin was given. The persistence of vaginal mucification indicated that the corpora lutea continued to function in the absence of the hypophysis under the influence of the injected hormone. Luteotrophin was extracted from pituitaries of sheep and was separated from follicle-stimulating and luteinizing hormones and from thyrotropin. Evidence is given to show that it is distinct from the growth and adrenotropic factors and that it is not responsible for the proliferation of mammary lobules in hypophysectomized rats. The role of this hormone in the regulation of corpus luteum function and the control of the reproductive cycle is discussed.

The author is indebted to Dr. E. Oppenheimer of the Ciba Pharmaceutical Co. Inc. for supplies of estradiol dipropionate, to Dr. J. A. Morrell of E. R. Squibb & Sons for the follutin used in these experiments and to Dr. L. Klein of Hoffmann-LaRoche Inc. for several batches of fresh pituitary glands.

REFERENCES

1. Fevold, H. L., F. L. Hisaw and S. L. Leonard: *Am. J. Physiol.* 97: 291. 1931.
2. Fevold, H. L., F. L. Hisaw, A. Hellbaum and R. Hertz: *Am. J. Physiol.* 104: 710. 1933.
3. Evans, H. M., K. Korpi, M. E. Simpson, R. I. Pencharz and D. H. Wonder: *Univ. Calif. Pub. in Anat.* 1: 255. 1936.
4. Fevold, H. L.: *Endocrinology* 24: 435. 1939.
5. Chow, B. F., R. O. Greep and H. B. van Dyke: *J. Endocrinol.* 1: 440. 1939.
6. Shedlovsky, T., A. Rothen, R. O. Greep, H. B. van Dyke and B. F. Chow: *Science* 92: 178. 1940.
7. Li, C. H., M. E. Simpson and H. M. Evans: *Science* 92: 355. 1940.
8. Makepeace, A. W., G. L. Weinstein and M. H. Friedman: *Am. J. Physiol.* 119: 812. 1937.
9. Dempsey, E.: *Am. J. Physiol.* 120: 126. 1937.
10. Astwood, E. B., and H. L. Fevold: *Am. J. Physiol.* 127: 192. 1939.
11. Astwood, E. B., and R. O. Greep: *Proc. Soc. Exper. Biol. & Med.* 38: 713. 1938.
12. Astwood, E. B., and R. O. Greep: *Science* 89: 31. 1939.
13. Dresel, I.: *Science* 82: 173. 1935.
14. Lahr, E. L., and O. Riddle: *Proc. Soc. Exper. Biol. & Med.* 34: 880. 1936.
15. Nathanson, I. T., and H. L. Fevold: *Endocrinology* 22: 86. 1938.
16. Lyons, W. R.: *Cold Spring Harbor Symp. Quant. Biol.* 5: 198. 1937.
17. Evans, H. M., M. E. Simpson and K. Turpeinen: *Anat. Rec.* (*Supplement* 3) 70: 26. 1938.
18. Rothchild, I., R. K. Meyer and M. A. Spielman: *Am. J. Physiol.* 128: 213. 1940.
19. Astwood, E. B.: *J. Endocrinol.* 1: 49. 1939.
20. Fevold, H. L., M. Lee, F. L. Hisaw and E. S. Cohn: *Endocrinology* 26: 999. 1940.
21. Li, C. H., W. R. Lyons and H. M. Evans: *J. Am. Chem. Soc.* 62: 2925. 1940.
22. Wolfe, J. M.: *Proc. Soc. Exper. Biol. & Med.* 32: 757. 1935.
23. Selye, H., J. B. Collip and D. L. Thomson: *Proc. Soc. Exper. Biol. & Med.* 32: 1377. 1935
24. Mazer C., S. L. Israel and B. J. Alpers: *Endocrinology* 20: 753. 1936.
25. Ellison, E. T., and J. C. Burch: *Endocrinology* 20: 746. 1936.
26. Merckel, C., and W. O. Nelson: *Anat. Rec.* 76: 391. 1940.
27. Selye, H., and S. M. Friedman: *Endocrinology* 27: 857. 1940.
28. Fevold, H. L.: *Cold Spring Harbor Symp. Quant. Biol.* 5: 93. 1937.
29. Greep, R. O.: *Endocrinology* 23: 154. 1938.
30. Westmann, A., and D. Jacobsohn: *Acta obst. et gynec. Scandinav.* 17: 1, 13. 1937.
31. Robson, J. M.: *J. Physiol.* 90: 435. 1937.
32. Williams, P. C.: *Nature* 145: 388. 1940.
33. Pencharz, R. I.: *Science* 91: 554. 1940.
34. Smith, P. E.: *Am. J. Anat.* 45: 205. 1930.
35. Corner, G. W.: *Am. J. Physiol.* 95: 43. 1930.
36. Gomez, E. T., and C. W. Turner: *Mo. Agr. Exper. Sta. Res. Bull.* 259: 1937.
37. Lewis, A. A., and C. W. Turner: *Mo. Agr. Exper. Sta. Res. Bull.* 310: 1939.
38. Pencharz, R. I., and J. A. Long: *Am. J. Anat.* 53: 117. 1933.

39. Selye, H., J. B. Collip and D. L. Thomson: *Proc. Soc. Exper. Biol. & Med.* 30: 589. 1933.
40. Liu, S. H., and R. L. Noble: *J. Endocrinol.* 1: 15. 1939.
41. Teel, H. M.: *Am. J. Physiol.* 79: 170. 1926.
42. Brouha, L.: *Proc. Soc. Exper. Biol. & Med.* 25: 488. 1928.
43. Cole, H. H.: *Am. J. Physiol.* 119: 704. 1937.
44. Fevold, H. L.: *Anat. Rec.* (*Supplement 2*) 73: 19. 1939.
45. Dempsey, E. W., R. Hertz and W. C. Young: *Am. J. Physiol.* 116: 201. 1936.
46. Astwood, E. B.: *Am. J. Physiol.* 126: 162. 1939.
47. Everett, J. W.: *Endocrinology* 27: 681. 1940.
48. Meyer, R. K., and R. Hertz: *Am. J. Physiol.* 120: 232. 1937.
49. Greep, R. O.: *Proc. Soc. Exper. Biol. & Med.* 34: 754. 1936.
50. Brooks, C. McC.: *Am. J. Physiol.* 121: 157. 1938.
51. Dempsey, E.: *Am. J. Physiol.* 126: 758. 1939.
52. Dempsey, E., and U. U. Uotila: *Endocrinology* 27: 573. 1940.
53. Uotila, U. U.: *Res. Publ. Ass. New. Ment. Dis.* 20: 580. 1940.
54. Brooks, C. McC., S. Bojar and W. G. Beadenkopf: *Endocrinology* 27: 873. 1940.

STUDIES ON PITUITARY IMPLANTS AND EXTRACTS IN ADRENALECTOMIZED RATS

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FOUR YEARS AGO, we (1) discussed the literature pertaining to the beneficial effect of the corpus luteum in adrenalectomized rats and in addition showed that pituitary grafts were very effective in prolonging life and maintaining body growth in adrenalectomized female rats. These beneficial results of pituitary grafts were absent when they were made in oöphorectomized and in male rats. Cavanaugh and Gaunt (2) confirmed our results regarding pituitary implants. They also showed that antuitrin-S and pituitary extracts would not prolong the life of adrenalectomized rats. Recently D'Amour and D'Amour (3) demonstrated some beneficial effects in female adrenalectomized rats treated with urine extracts, such as gonadin and follutein. In this paper, we present additional data on pituitary grafts and extracts in adrenalectomized rats.

METHODS

Female albino rats were used. The skin of the operative area was shaved and sterilized with alcohol. Adrenalectomy was performed in 2 stages, at 25 and 30 days of age. One castrated rat pituitary was placed in the abdomen at the time each adrenal was removed. Following this, a pituitary implant was made in alternate hind legs at 5-day intervals up to 30 days after adrenalectomy. The controls, litter mates of the grafted rats, were adrenalectomized in the same way. The pituitary extract[1] (15 R.U.) was given in 1 cc. of normal salt solution twice daily. One injection was made intraperitoneally each morning and a second injection subcutaneously in the afternoon; The rats lived at all times in a room having a constant average temperature of 26°C. with a variation of 2°C. They were fed Purina Dog Chow and water.

RESULTS

Effect on body weight. The rats receiving the pituitary implants grew at a consistently faster rate than the normal controls and far better than the adrenalectomized untreated litter mates. Figure 1 (*A*, *B*, *C*) depicts these 3 groups for a period of 30 days after adrenalectomy. It was evident even 5 days after the first implant (*Group A*, fig. 1) that these rats were growing at a faster rate than the other groups. In contrast to this, the weight curve of untreated rats usually began to flatten after removal of the first adrenal and was slightly below normal by the time the second adrenal was removed (fig. 1) (4). It was previously noted, without giving any significance to it, that adrenalectomized rats treated with pituitary implants were heavier than normal female rats (1). This adds evidence in support of the growth curves shown in figure 1, that the rats with pituitary implants grew faster than normal. The growth curve was

Received for publication August 12, 1940.

[1] We are grateful to C. A. Oclassen of Ayerst, McKenna & Harrison for the pituitary gonadotropic extract of sheep used in these experiments.

distorted and of little value beyond 30 days after adrenalectomy due to the fact that the emaciated and smaller rats died first. Those remaining were larger and grew more normally so that by the end of 20 to 30 days the growth curves for the treated and untreated adrenalectomized rats were similar to each other and also to the growth curve for normal rats (fig. 1) (1, 4, 5).

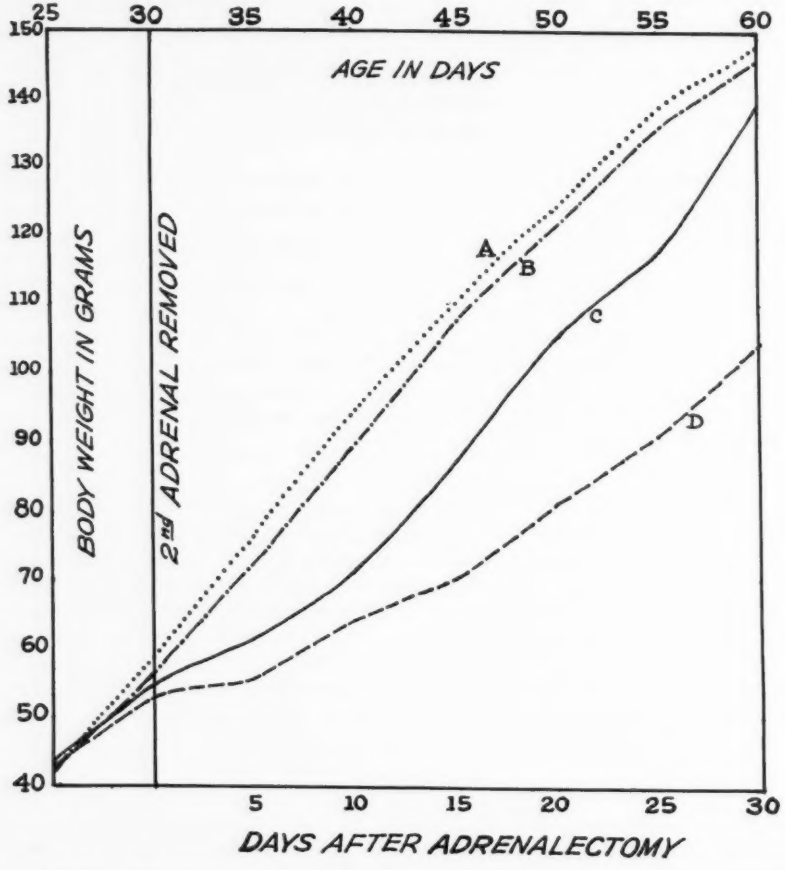

Fig. 1. Growth curves for female albino rats from 25 to 60 days of age. *A*, 25 adrenalectomized rats given one castrated rat pituitary at 5-day intervals. *B*, 25 normal rats. *C*, 25 adrenalectomized rats not treated. *D*, 20 adrenalectomized rat treated with 30 r.u. of pituitary gonadotropic extract daily.

The beneficial effect of pituitary extract on body weight was not evident in these experiments. In doses of 30 r.u. daily the curve for the mean body weight was even below the untreated adrenalectomized controls (fig. 1 *C*, *D*). This may have been due either to some deleterious properties of the extract or to the fact that even the small and emaciated rats lived longer (fig. 2), thus lowering the growth curve as contrasted to the adrenalectomized controls in which the death rate was much higher. The ovaries in this group were not as large as those obtained from the recipients of the pituitary grafts. Frequently the dead rats were badly eaten, but where the ovaries were

present, they were weighed. In the rats given pituitary implants and extract (fig. 1, *Group A, D*), the mean ovarian weights obtained from those dying between 5 and 30 days after adrenalectomy were 122 and 85 mg. respectively.

Effect of length of life. The power of pituitary implants to prolong life in adrenalec-

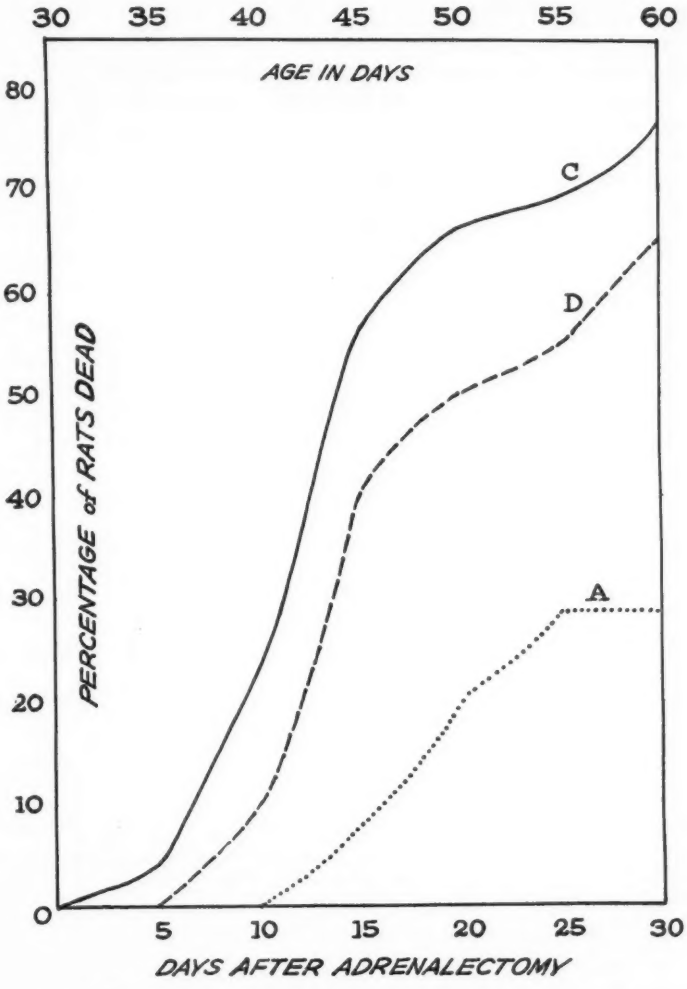

Fig. 2. THE PERCENTAGE OF RATS DEAD UP TO 30 DAYS AFTER ADRENALECTOMY. *A*, implanted; *C*, no treatment; *D*, gonadotropic extract as in figure 1.

tomized rats was also very gratifying. The curve representing the percentage of rats dead shows clearly the beneficial effect of the implants (fig. 2). Only 28%, in this group, were dead by the end of 30 days as contrasted to 76% in the controls and 65% in those treated with pituitary extract (fig. 1, *A*, *C*, *D*). This was interpreted as reflecting the effect of the secretion from the corpora lutea which was undoubtedly

more abundant following pituitary implants than with injection of pituitary extract.

So far a procedure has not been devised to keep adrenalectomized rats alive indefinitely with pituitary grafts. Attempts (10 rats) to also graft additional ovaries in the muscles of these recipients at the time the pituitary implants were made have not yielded results any more beneficial than those obtained without the extra ovarian tissue.

DISCUSSION

Estrin in small amounts was found useless in adrenalectomized rats (4, 6) and in larger amounts, definitely harmful (2). Since estrin production was concomitant with corpora lutea secretion, in these experiments the beneficial effects of the pituitary implants were of even greater significance. The amount of progesterone necessary to keep adrenalectomized rats alive was shown to be 1 mg. or more daily (3; citations by Emery and Greco, 5). Some rats (fig. 1) were not able to obtain this amount for long periods from their own corpora lutea even though new stimuli were given in the form of pituitary implants. Previous data showed that new corpora were more useful than old corpora (1) and that a pituitary implant would supply hormones for at least eight days (7).

The effect of the pituitary implants on body growth is highly successful; as shown in figure 1 the mean body weight is consistently above that of normal rats. It is known that the secretion from the corpora lutea keeps adrenalectomized rats alive, and it is presumed that the growth and other hormones, in addition to the gonadotropic from the implanted tissue, help induce the phenomenal growth that is seen.

SUMMARY

The previous studies on the beneficial effect of pituitary implants in adrenalectomized rats are confirmed and extended. In addition, the data described support the following.

Implants of pituitary glands of castrated rats are capable of keeping adrenalectomized female rats alive and growing at a rate above normal.

Extracts of the pituitary gland prolong life but are far inferior to pituitary implants.

Ovarian tissue implanted in the muscles simultaneously with implants are not beneficial to adrenalectomized rats.

The hormones which are probably involved are discussed.

REFERENCES

1. Emery, F. E., and E. L., Schwabe: *Endocrinology* 20: 550. 1936.
2. Cavanaugh, C., J., and R. Gaunt: *Proc. Soc. Exper. Biol. & Med.* 37: 226. 1937.
3. D'Amour, M. C., and F. E. D'Amour: *Proc. Soc. Exper. Biol. & Med.* 40: 417. 1939.
4. Schwabe, E. L., and F. E. Emery: *Proc. Soc. Exper. Biol. & Med.* 40: 383. 1939.
5. Emery, F. E., and P. A. Greco: *Endocrinology* 27: 473. 1940.
6. Martin, S. J.: *Am. J. Physiol.* 100: 180. 1932.
7. Emery, F. E.: *Anat. Rec.* 66: 253. 1936.

THE PRESENCE AND CHEMICAL DETERMINATION OF ADRENO-CORTICAL ('AC') COMPOUNDS IN THE BLOOD[1]

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THE STUDY of suprarenal physiology and pathology has always been considerably handicapped by the lack of reliable chemical methods for determining the level of suprarenal hormones in the body fluids and tissues. Many previously described methods proved too unspecific and inconsistent in their results to be of practical value. The method of Shaw (1) which is partly based upon that of Whitehorn (2) and which was originally designed for the determination of adrenalin in organs, seemed at first to yield good results also in blood according to Bloor (3). However, investigations of my own and, according to a personal communication from Dr. Bloor, his experience also, have revealed fairly irregular results by the original procedure and by various modifications which were tried. Furthermore, most 'adrenalin' values obtained in the blood with this method were considerably greater than could be probably accounted for by adrenalin alone, and Shaw himself admitted in a personal communication the interference of an "unknown substance" in blood.

The primary purpose of the present investigation was an attempt to improve Shaw's original technic regarding its quantitative reliability for blood determinations and to identify the interfering substance or substances.

Modification of Shaw's Method

The following points proved useful to obtain accurate and consistent values in the blood.

1). It was found essential to adjust the pH of the protein-free blood filtrate carefully within a range of 3.8 to 4.2 before the first adsorption of accompanying substances by $Al(OH)_3$. Higher degrees of acidity raised the final results in a steep curve (fig. 1). Stronger alkalinity up to pH 5.6 is of no particular importance but was routinely avoided. A Beckman pH-meter was used.

2). Boiling of the final solution with the arsenomolybdic reagent was continued for 10 minutes instead of 5, as suggested by Bloor (3).

3). The aqueous solution of phenolphthalein (0.1 : 100) should be prepared fresh each day. If older than 5 to 6 hours it is likely to increase the intensity of the blue color, even if only one drop is used.

4). In contrast to Shaw's directions blanks were not used to determine the final results. Instead, a standard curve was constructed from readings obtained with various adrenalin dilutions which were compared with a solution of $1 : 10^7$. This curve proved sufficiently constant on many checks and was used for direct readings (fig. 2).

With these precautions satisfactorily consistent results were obtained in blood, for instance in one sample of blood 130, 124, 130, 130 color units per cc. One color unit equals the colorimetric effect of one mγ (=milligamma=billionths of a gram)

Received for publication July 5, 1940.
[1] This study was aided by a grant from the Rockefeller Foundation.

of adrenalin per cubic centimeter of the standard solution. Adrenalin which had been added to the deproteinized filtrate before analysis, could be recovered, for instance:

Blood alone: ..... 176 color units per cc.
Blood + 20 mγ/cc. adrenalin: 191 = 176 + 15 color units per cc.
Blood + 50 mγ/cc. adrenalin: 227 = 176 + 51 color units per cc.

In 51 human individuals the values found per cc. of blood ranged between 60 and 222 with an average of 156 color units per cc. These figures are much higher than the few given by Shaw (16 to 20 mγ/cc.) and much lower than some of Tietz et al. (4) (0 to 2000 mγ/cc.). They agree better with those found by Bloor (180 to 240 mγ/cc., according to a personal communication). Repeated determinations in normal persons on different days yielded the following results.

			color units per cc.
W.R.	August 5	1939	113
	August 7	1939	132
	August 10	1939	126
	August 20	1939	83
	August 29	1939	126
	October 23	1939	164
	May 9	1940	160
L.W.	August 8	1939	140
	August 21	1939	162
P.J.	August 23	1939	203
	September 9	1939	186

It is necessary that all manipulations and time measurements be executed with the greatest possible accuracy and uniformity. An outline of the procedure is given below. It differs only in a few but essential details from Shaw's directions. The preparation of the reagents is described in Shaw's original paper (1).

1). Withdraw at least 4 cc. of blood from an arm vein (as a rule 10 cc. were taken). Place the blood immediately into a test tube containing an equal amount of 10% trichloracetic acid and shake vigorously. Remove stopper for a moment to allow gas to escape.

2). Filter the mixture through a paper filter into a graduated cylinder and wash the residue on the filter with more trichloracetic acid until a filtrate volume thrice that of the blood used is reached (for instance a filtrate of 30 cc. for 10 cc. of blood).

3). Stir the filtrate well and transfer 6 cc. into each of 2 graduated 15 cc. centrifuge tubes (for duplicate determinations) so that each of them contains the equivalent of 2 cc. of blood. Add one drop of fresh aqueous phenolphthalein solution (0.1 gm.: 100 cc. N/100 NaOH) which is not older than 5 to 6 hours.

4). Adjust the pH to 3.8 to 4.2. This is done by first adding approximately 1.5 cc. of 4% NaOH from a burette and then continuing cautiously drop by drop while stirring with a glass rod until the red color in the uppermost portion of the fluid persists for about a second. Then shift to another burette, containing 0.8% NaOH and add this with continuous stirring drop by drop, finally fractions of drops until a light pink color persists.

Add with a graduated pipette about 0.2 cc. of N/5 H_2SO_4. Stir well with glass rod and determine pH. If it is more alkaline than 4.2, add more H_2SO_4 in small amounts.

5). Add 2 cc. of $Al(OH)_3$ reagent, stir well and centrifuge (3000 revolutions per minute) for two minutes.

6). Transfer supernatant clear fluid into another centrifuge tube, add to it again 2 cc. of the $AL(OH)_3$ reagent and one drop of phenolphthalein. Add NaOH (4%) until a distinctly pink color appears (about 5 to 6 drops). Stir well. Centrifuge again for 2 minutes and discard the supernatant fluid. Add 3 cc. of slightly alkalized water to the sediment. Stir the sediment thoroughly until all lumps are dispersed. Centrifuge again for 2 minutes and discard the supernatant fluid. Add 2 cc. of distilled water to the sediment. Stir up thoroughly.

7). Prepare sulfite reagent by placing 2 cc. of Na_2SO_3 solution (not older than 2 days) into 14 cc. of H_2SO_4 (1:1) and stir the mixture. This combined reagent must not be older than one-half hour.

8). Put exactly 0.7 cc. of arsenomolybdate reagent into a graduated centrifuge tube and place the tube in boiling water.

9). Exactly 3 minutes later add 0.35 cc. of 4% NaOH to the test suspension (containing the $Al(OH)_3$ plus adsorbate) and stir a few seconds. The suspended $Al(OH)_3$ need not go completely into solution.

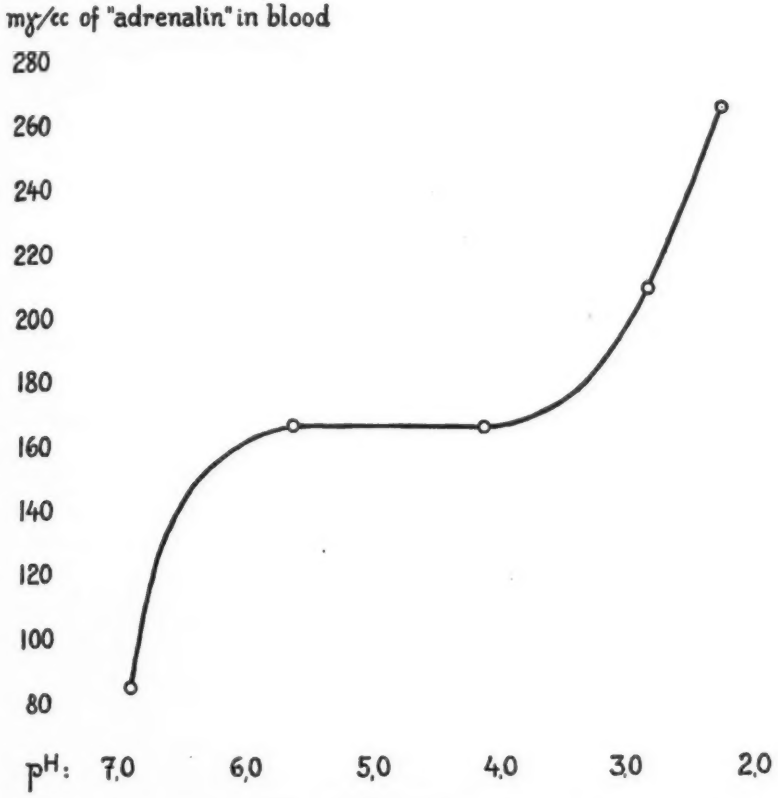

Fig. 1. The influence of the pH at which the first adsorption by $Al(OH)_3$ takes place, upon the "adrenalin" readings of a blood sample the "adrenalin" content of which was found to be 169 mγ/cc. at a pH from 4.2 to 5.6.

10). Exactly 2 minutes after adding the NaOH add 2 cc. of the sulfite reagent stir quickly with a glass rod until solution is clear and pour it into the heated arsenomolybdic reagent. Heating of the mixture in boiling water is continued for exactly 10 minutes longer.

11). Hereafter place the test tube in cold water, add distilled water to 5.5 cc. and transfer contents after 10 minutes at the earliest into a colorimeter cup for comparison with a similarly prepared standard adrenalin solution.

12). The latter is prepared by diluting 2 cc. of an adrenalin solution of $1:10^7$ concentration which should be standing for at least half an hour but not longer than a few hours, in a centrifuge tube with 6 cc. of distilled water. Two cc. of the $Al(OH)_3$ reagent and one drop of phenolphthalein are added. Alkalinization to distinctly pink color takes place with about 5

drops of 4% NaOH. Further procedure as above (No. 6, etc.). The first adsorption by $Al(OH)_3$ is unnecessary in this case because all of the pure adrenalin ia adsorbed by $Al(OH)_3$ in an alkaline medium.

13). Using a standard type Bausch & Lomb colorimeter (Dubosq system) with glass cups (no metal) the standard solution is put in one cup at position 10.0 and the reading of the test solution taken from the other scale.

14). Adrenalin equivalents (color units) of the test solution are determined directly by comparison of the colorimeter readings with the standard adrenalin curve (fig. 2). One color unit corresponds to one mγ of adrenalin.

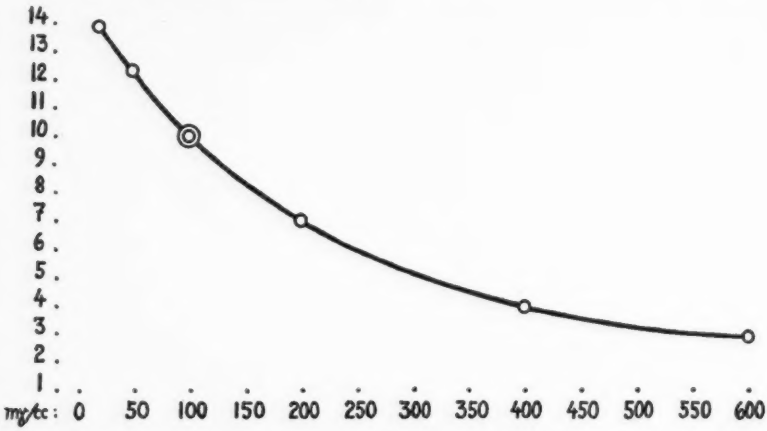

Fig. 2. ADRENALIN STANDARD CURVE CONSTRUCTED FROM COLORIMETRIC READINGS WHICH WERE OBTAINED BY COMPARING VARIOUS ADRENALIN DILUTIONS (20, 50, 100, 200, 400 and 600 mγ/cc.) with an adrenalin solution of 1:10 million (100 mγ:cc.). The latter was constantly kept at point 10.0 of the colorimeter scale.

Shaw's Specificity Test for Adrenalin

Shaw (1) stated that adrenalin differs specifically from all other substances which give a similar color reaction, in that its color effect is 2 to 3½ times more intensive with alkalinization (see above No. 9) than with addition of one drop of N/1 H_2SO_4. This could be confirmed with a ratio of acid:alkaline readings equaling 1:3.5 for pure adrenalin. However, in using blood filtrates instead of adrenalin solutions this ratio was found to be quite different. According to Shaw (1) it is 1:1.2 for human blood. In my own human material it varied between 1:0.7 and 1:1.87. In any event blood never gives a specificity test typical for adrenalin. This is further evidence of the interference of other substances different from genuine adrenalin.

The absolute values of 'adrenalin' found with different methods by different authors vary in extremely wide ranges.

'Adrenalin' in normal human blood

	mγ/cc.
Hülse (5)	1
Stewart (6)	2– 10
Shaw (1)	16– 20
Adler (7)	100
Trendelenburg (8)	100
Bloor (3)	180– 240
Kuré et al. (9)	500
Konschegg and Monauni (10)	500

Stuber et al. (11)	1000
Tietz et al. (4)	0–2000
Fraenkel and Allers (12)	2500
Viale (13)	2500
Crocetta and Dogliotti (14)	2500
Giordano and Zeglio (15)	300–3200

Substances Which Give an Adrenalin-like Color Reaction

Shaw (1) has examined several sympathicomimetic and other substances in regard to their blue reaction with the arsenomolybdate reagent. None of them could compare with adrenalin in color intensity and none of them, except p-sympatol, gave the adrenalin specificity test.

Concerning the adsorbability of the substances examined, Shaw mentions only the complete and exclusive adsorption of adrenalin by $Al(OH)_3$ in an alkaline medium

TABLE 1

Substance	Amount per cc.	Color effect compared with adrenalin	Ratio of color. Acid:alkaline readings	Adsorption by $Al(OH)_3$ at pH	
				3.5–4.0	8.5
Adrenalin	0.1γ	1:1	1:3.5	0	100%
Ascorbic acid	50γ	1:320	1:0.3	0	100%
Glutathione	500γ	1:2500	1:1.1	100%	40%[1]
Vitamin D_2	25γ	1:77	1:1.0	63%[2]	48%[1]

[1] These figures indicate the percentage of adsorbed material if adsorption at pH 8.5 was attempted directly without previous adsorption in an acid medium.

[2] The supernatant fluid of the centrifuged acid adsorbate was found free of color giving substance. It seems that the color giving quality of vitamin D_2 is reduced through acidification to a pH of 3.5.

and the complete adsorption of glutathione in an acid medium. Both of these statements I could confirm.

Shaw also claims a non-adsorbability of ascorbic acid in an alkaline medium. In this latter respect my own findings differ from his (see below).

In a further series of my own experiments I found the following substances did not give any appreciable color reaction with the arsenonomolybdate reagent: dextrose, urea, creatinine, leucin, tyrosin, glycocoll, tyramin, histidin, cystein, histamin, acetylcholin, thiamin, nicotinic acid, riboflavin, pitressin, pitocin, acetone, beta-oxybutyric acid, lactic acid, cholesterol, androsterone, alcohol, nicotin, cocain and dialyzed iron.

Among those substances which gave a positive color reaction (table 1) glutathione and crystallized vitamin D_2 were the only ones which were adsorbed by $AL(OH)_3$ in an acid medium and thus can be expected to be removed from the blood filtrates through the first adsorption at a pH around 4.0.

Whether and what other color giving substances are eliminated by this first adsorption is still an open question.

Regarding ascorbic acid which, according to Shaw, is not at all adsorbed, I found a different behavior, in that 50γ per cc. of ascorbic acid was not adsorbed at a pH around 4.0 but appeared 100% in the second (alkaline) adsorbate, thus directly interfering with the 'adrenalin' reaction of blood filtrates. However, the color intensity is fairly weak (1/500 of that of adrenalin according to Shaw, 1/320 according to my own findings). The usual concentration of ascorbic acid in the blood ranges between 5 and 10γ per cc. according to Stepp, Kühnau and Schröder (16), between 5 and 25γ per cc. according to Farmer and Abt (17), between 6 and 15γ per cc. according to Stephens and Hawley (18). This would allow ascorbic acid to take part in the final reaction of normal blood filtrates with a resulting color intensity of 15 up to a maxi-

mum of about 80 color units per cc. With the average normal readings being 156 color units per cc. (see page 320), ascorbic acid would form from 10 up to 50% of the readings for normal blood 'adrenalin.' Under exceptional circumstances, as in leukemia, the blood level of ascorbic acid is markedly increased due to the ascorbic acid content of the white blood cells (18). I have accordingly found extremely high figures for blood 'adrenalin,' in fact, the highest which I have ever observed, in leukemia (for instance 720 color units per cc. in one case with 689,000 leukocytes; 305 color units per cc. in another one with 167,000 leukocytes). The specificity test (ratio acid:alkaline results, see page 321) for ascorbic acid is the opposite of that for adrenalin, namely 1:0.3 instead of 1:3.5. Therefore it seems probable that the lack of a positive adrenalin specificity test in blood filtrates is partly due to the interfering presence of ascorbic acid.

Table 2 shows a group of substances which gave an adrenalin-like blue reaction but were not or probably were not adsorbed by $Al(OH)_3$.

Although the non-adsorbability of oleinic acid, lecithin and cephalin could not be directly demonstrated, due to the formation of disturbing precipitates, it is highly

TABLE 2

Substance	Amount per cc.	Color effect compared with adrenalin	Ratio of color. Acid:alkaline readings	Adsorption by $Al(OH)_3$ at pH	
				3.5–4.0	8.5
Uric acid	500γ	1:300	1:1.3	o	o
Oleinic acid	500γ	1:130[1]	1:4.8[1]	?	?
Lecithin	600γ	1:6000[1]	1:1.0[1]	?	?
Cephalin	600γ	1:2000[1]	1:1.0[1]	?	?
Thyroxine	60γ	1:155	1:1.1	o	o
Insulin	40 U	+[1]	1:2.5[1]	?	?
Ergotamin	50γ	1:150	1:1.1	o	o
Strophantin K	20γ	1:200	1:1.0	o	o

[1] These figures are only approximate due to the formation of precipitates which interfered with colorimeter readings. For the same reason the adsorbability could not be reliably tested.

improbable that they participate in the blood 'adrenalin' results in view of *a*) their weak color intensity, *b*) the absence of analogous precipitates in blood filtrates, and *c*) the fact that in clinical cases with extreme hyperlipemia (diabetes) no abnormally intense color reactions were observed.

Another group of substances comprises cortical and gonadal hormonal sterols (table 3) all of which, except cholesterol and androsterone, gave an adrenalin-like blue reaction. None of them was adsorbed by $Al(OH)_3$ either in an acid or alkaline medium and their specificity ratio (acid:alkaline results) was different from that of adrenalin in all instances.

The strongest color reaction was displayed by two cortical sterols (corticosterone and Kendall's compound *E*). In comparison to adrenalin it was 1/41 and 1/42 respectively; the next in color intensity were desoxycorticosterone acetate, progesterone and α-estradiol (1/71–75); the weakest color effect (1/100) was produced by testosterone and by the synthetic Δ-4 and rostenedione (Ciba). All of these substances as also some of the aforementioned, were first dissolved in a small amount of alcohol and the alcoholic solution transferred into water where, in some instances, a fine precipitation formed.

Formation of Sterol-Adrenalin Compounds in Vitro

According to Konschegg (19) most of the adrenalin within the adrenal glands is bound to some lipoid substances. The amount of free adrenalin in acid suprarenal extracts increases slowly through disintegration of these compounds. The speed of ap-

pearance of free adrenalin is proportional to the degree of acidity of the surrounding medium. For instance, the same amount of adrenalin was liberated by 2% acetic acid in 6 to 8 days as by 10% acetic acid in one day. On the other hand, if adrenalin was mixed with aqueous suspensions of lipoids which had been extracted from organs or from blood serum, it disappeared gradually so that after about two weeks no more free adrenalin was detectable by the method of Zanfrognini. This process is prevented through the presence of even minimal amounts of acids. Konschegg (19) did not attempt to specify the chemical nature of those lipoids which are apt to bind adrenalin. He emphasized, however, that cholesterol is not among them. Konschegg's statements are in accordance with those of Koehler and Eichelberger (20) who found in the suprarenal cortex lipoid substances which originally seemed to contain no adrenalin

TABLE 3

Substance	Amount per cc.	Color effect compared with adrenalin	Ratio of color. Acid : alkaline readings	Adsorption by $A.(OH)_3$ at pH	
				3.5–4.0	8.5
Cholesterol	1000γ	0	—	—	0
Corticosterone	40γ	1:42	1:0.9	0	0
Cortical compound E (Kendall)	40γ	1:41	1:0.9	0	0
Desoxycorticosterone acetate	20γ	1:73	1:1.2	0	0
Progesterone	20γ	1:75	1:1.5	0	0
α-estradiol	20γ	1:71	1:1.7	0	0
Testosterone	10γ	1:100	1:1.4	0	0
Δ-4 androstenedione	10γ	1:100	1:1.1	0	0
Androsterone	20γ	0	—	—	—

but from which adrenalin could be split off through acids. Similar findings have been reported by Kendall et al. (21). Abelous, Soulié and Toujan (22) observed a considerably increasing amount of adrenalin in isolated cortical material which was kept for 24 hours at a temperature of 37°C.

The findings of the authors mentioned above suggested two possibilities in regard to the interpretation of the comparatively large 'adrenalin' figures obtained with Shaw's method in blood.

a). Splitting off of otherwise concealed extra amounts of adrenalin from adrenalin-lipoid compounds through the procedure of deproteinization with trichloracetic acid at a pH of 1.5. This possibility could be ruled out by replacement of the trichloracetic acid by $ZnSO_4$ (deproteinization method of Somogyi at a pH of 7.0) which did not alter the results.

b). It seemed possible that color giving (but by themselves unadsorbable) cortical and other sterols might be adsorbed by $Al(OH)_3$ and thus appear in the colorimetric results after compound formation with adrenalin.

In order to test this latter assumption adrenalin was added to aqueous solutions and suspensions of such sterols which had been found to give a blue color reaction with Shaw's arsenomolybdate reagent. The mixtures of sterols with adrenalin were kept at various pH levels for varying durations of time, either at room temperature or in an incubator at 38°C. In order to avoid misinterpretations, both the sterols and adrenalin were examined separately under analogous conditions. The sterols alone (without adrenalin) remained unadsorbable under all circumstances tested. Adrenalin alone remained practically unchanged, both concerning its color intensity and the behavior of the specificity test, if kept at a pH of about 7.5. With increasing alkalinity of the surrounding medium up to a pH of 10.7 the color intensity of adrenalin decreased somewhat. The color effect of some sterol-adrenalin mixtures examined directly without adsorption remained practically unchanged during incubation of as much as 44 hours duration, for instance:

a) 20γ desoxycorticosterone acetate.....270 color units
b) 100 mγ adrenalin.....100 color units
c) Mixture of a+b (fresh).....381 color units
d) Mixture of a+b (after 44 hours at pH 7.2 and 38°C).....374 color units

In view of the above findings it would seem justifiable to assume that the formation of adsorbable sterol-adrenalin compounds had occurred, if the adsorbate of sterol-adrenalin mixtures, each containing 100 mγ./cc. of adrenalin, yielded higher figures

TABLE 4

Sterols	Amount	Adrenalin added	pH adjusted to	Duration of exposure	Temperature	Amount of sterol adsorbed per 100 mγ of adrenalin[1]	Color ratio. Acid:alkaline readings of adsorbed compounds
	γ	mγ		hr.	°C.	γ	
Corticosterone	20	100	7.5	1½	38	0	
	20	100	7.5	20	38	1.9	1:2.8
	20	100	7.5	40	38	1.6	
Compound *E* (Kendall)	20	100	7.4	20	38	0	
Desoxycorticosterone acetate	20	100	7.2	¼	22	0	
	20	100	7.2	20	20	2.6	
	20	100	7.4	20	38	17.6[2]	1:1.1
	20	100	9.5	24	38	19.0	
	20	100	10.8	½	22	6.3	
	20	100	10.7	24	22	7.3	
Progesterone	20	100	7.5	44	38	3.8	
	20	100	9.5	24	38	19.0	
α-estradiol	20	100	7.5	1½	38	0	
	20	100	7.5	20	38	8.5	1:1.1
	20	100	7.9	40	38	8.0	
Testosterone	20	100	7.4	20	38	8.6	1:0.8
Δ-4 androstenedione	20	100	7.4	20	38	2.9	1:1.7

[1] Calculated from increase in color beyond 100 mγ of adsorbate obtained at pH 8.5.
[2] This was the maximal result among several repetitions of the same experimental order, some of which had given lower figures (e.g. 3.0γ).

than 100 color units per cc. As the color giving intensity of various sterols was known (see table 3), the absolute amounts adsorbed by $Al(OH)_3$ with adrenalin could be estimated from the increase in color effect beyond 100 color units per cc.

Table 4 shows the calculated amounts of a number of cortical and gonadal sterols which had been adsorbed by $Al(OH)_3$ in a medium of pH 8.5 with adrenalin. It was found that the assumed compound formation did not take place immediately. At a pH around 7.5, which is near to that of body fluids, it was not noticeable after 1½ hours but the maximum was reached within about 20 hours, after which it did not increase further. Body temperature (38°C.) seemed to have an enhancing effect upon compound formation in comparison with room temperature. Compound formation was markedly speeded up in an alkaline medium (for instance almost to a maximum within one-half hour at a pH of 10.8), although too intense an alkalinization interfered somewhat with the color giving effect.

Subsequent acidification to a pH of 3.0 of an alkaline desoxycorticosterone-adrenalin mixture which had shown an adsorption of 7.3γ of the sterol per 100 mγ adrenalin, reabolished the adsorbability of the sterol completely.

At a pH of approximately 7.5 and at body temperature, thus under conditions similar to those prevailing in the living organism, the order of affinity of cortical sterols to adrenalin seemed to be approximately the following.

Desoxycorticosterone (17.6γ per 100 mγ adrenalin)
Progesterone (3.8γ per 100 mγ adrenalin)
Corticosterone (1.9γ per 100 mγ adrenalin)
Compound *E* (Kendall) (0γ per 100 mγ adrenalin).

However, these quantitative results have to be regarded with caution, as the pH, even if adjusted exactly to 7.5 in the fresh mixture, tends to shift gradually toward the acid side, which apparently interferes with the compound formation, influences precipitation and, therefore, makes the results rather irregular.

The specificity tests (ratio acid: alkaline results, see page 321) of those amounts of the synthetic cortical sterol-adrenalin compounds which were obtained through adsorption by $Al(OH)_3$ fell into the same range as the specificity ratios of blood filtrates:

Specificity ratio of pure adrenalin:.....1:3.5
Specificity ratio of sterol-adrenalin compounds:.....1:0.8–1:1.7
Specificity ratio of normal blood filtrates:.....1:0.7–1:1.87

Components Contributing to the Results Obtained With the Modified Method of Shaw

From the findings discussed above it appears evident that the results obtained with Shaw's method comprise not only free adrenalin (if there is any in the blood at all) but also ascorbic acid and such amounts of color giving sterols as are tied up with adrenalin. Whether only cortical sterols, such as corticosterone and desoxycorticosterone, or also gonadal sterols, such as estradiol and testosterone, are represented among the adsorbable sterol-adrenalin compounds of the blood, cannot be decided at present. However, in view of the slowness of the sterol-adrenalin compound forming process, it seems reasonable to assume that it will take place to a larger extent within the suprarenal glands where cortical sterols and adrenalin mingle continuously in high concentrations, than within the blood stream where adrenalin and gonadal sterols meet in diluted form.

The suprarenal cortex contains a large number of sterols (23, 24) of different functional importance, only three of which I could examine concerning their ability to form compounds with adrenalin. Progesterone has also been found in the cortex by Beall and Reichstein (25). It is not only biologically and structurally most closely related to desoxycorticosterone but also very similar to the latter in regard to color intensity, acid-alkali ratio and degree of adsorbability with adrenalin.

Cholesterol, which might have escaped detection in the blood filtrates because of its inability to give the blue color reaction, was found not to be present in the adsorbate by $Al(OH)_3$ in using the Liebermann-Burchard qualitative test. Furthermore, the blood of a patient with a high cholesterinemia yielded a normal colorimetric result.

There is ample morphological, clinical and experimental evidence of a very close functional cooperation between suprarenal cortex and medulla (19, 26–32) and for the sake of brevity I want to suggest the term 'adreno-cortical compounds' or 'AC' for the sterol-adrenalin compounds which are present in the blood and which can be detected with the modified method of Shaw.

My findings support Konschegg's statements concerning the presence of 'adrenalin-lipoid' compounds in the blood, their synthesis in vitro, their disintegration through acids, and the non-participation of cholesterol.

Both Konschegg and I have done experimental work with alcoholic lipoid extracts of the adrenal glands and of human serum. These extracts were found to cause vasoconstriction (10, 19), general nervous reactions, elevation of the blood sugar level and an enhancement of cholesterol atheromatosis in rabbits (32). In order to establish a possible relationship between those biologically powerful alcoholic 'lipoid' extracts of suprarenal glands and blood and the substances found in the blood by chemical means, I examined alcoholic extracts of human blood in the following manner: 20 cc. of blood were added to 20 cc. of absolute alcohol, shaken, spread on a plate and dried for 48 hours in an incubator at 38°C. The dry substance was pulverized and extracted with 120 cc. of absolute alcohol, both cold (through frequent washing on a filter) and hot (in the Soxhlet apparatus). The filtrates were slowly evaporated and the fatty residue was dissolved in low boiling petrol ether (30–35°). Forty cc. of 10% trichloracetic acid and 20 cc. of distilled water were added, the mixture was shaken and the supernatant petroleum ether was then evaporated. The remaining fluid was filtered and the filtrate treated in the same way as the original deproteinized blood filtrates. It was found that cold alcoholic extraction did not yield any appreciable amounts of color giving material while the entire amount of the latter was recovered through hot alcoholic extraction; for instance 138 color units per cc. were found in the alcoholic extract of a blood specimen which yielded 135 color units per cc. with the ordinary procedure. In some instances the results obtained with hot alcohol extraction appeared even somewhat higher.

On the other hand, attempts to extract the color giving substance with alcohol from the $Al(OH)_3$ adsorbate, in which it was contained, were to no avail.

The presence of the entire color giving material in hot alcoholic blood extracts proves the alcohol solubility of this material and suggests that it is at least partially identical with the biologically active constituents in both Konschegg's and my alcoholic blood extracts for which I had suggested the German term "Nebennieren-lipoid-Adrenalin-(NLA)-Komplexe."

The participation of a variety of substances (adrenalin, adrenalin-bound sterols, ascorbic acid) in the results obtained with Shaw's modified method does not allow an accurate estimation of these individual constituents. However, the behavior of the specificity test permits a rough evaluation of their quantitative distribution within the bulk of color giving material. Adrenalin with an acid:alkaline ratio of 1:3.5 and ascorbic acid with a ratio of 1:0.3 more or less counteract each other's effect upon the ratio of the total blood findings. The ratio of desoxycorticosterone which seems to be bound to adrenalin in particularly large amounts, is 1:1.1. Thus, if in a blood filtrate a low ratio is found, as for instance 1:0.9, it suggests a relatively strong participation of ascorbic acid, while a ratio of for instance 1:1.2 may indicate a stronger participation of adrenalin beside less noticeable changes in the amounts of sterols, for instance:

	Acid determination giving color units:	Alkaline determination giving color units:	Ratio
Adrenalin	11	38	1:3.5
Ascorbic acid	18	6	1:0.3
Sterols	80	88	1:1.1
Sum	109	132	1:1.2
	or:		
Adrenalin	3	10	1:3.5
Ascorbic acid	30	10	1:0.3
Sterols	101	112	1:1.1
Sum	134	132	1:0.9

This is, of course, only an arbitrarily constructed example, intended to demonstrate the possibility of the same final result (obtained with the routine alkaline treat-

ment) being due to various distributions of its constituents. It furthermore shows the eventual possibility of drawing some very rough quantitative conclusions concerning the relative proportion of these constituents from the ratio of acid:alkaline readings. Compound formation of adrenalin with some gonadal sterols seems to cause more complicated changes of the acid:alkaline ratio.

Results obtained with Shaw's modified method under various clinical and experimental conditions will be published in a series of other papers. The functional and pharmacodynamic significance of compound formation of adrenalin with various hormonal sterols will be studied separately.

DISCUSSION

Shaw's method for the determination of 'adrenalin' in blood was slightly modified in order to improve its accuracy. It reveals figures which have been found to be the sum of the effects of a number of substances giving the characteristic blue reaction. Beside free adrenalin which, if at all present, forms only a minor fraction of the results, ascorbic acid apparently accounts for another fraction of their total amount. The bulk of the final readings, however, seems to be due to the presence of compounds made up of sterols (in particular desoxycorticosterone) which are bound to adrenalin. The term 'adrenocortical compounds' or 'AC' is suggested. The above conclusion which is in accordance with Konschegg's findings concerning the presence of 'lipoid-adrenalin compounds' in the blood, is based upon the following facts.

A number of sterols give a comparatively strong blue reaction similar to that of adrenalin, if brought directly into contact with the arsenomolybdate reagent which is used in Shaw's procedure. On the other hand, they are not adsorbed by $Al(OH)_3$ in contrast to all substances which participate in the final results obtained with Shaw's method in blood. However, adsorption of these sterols by $Al(OH)_3$ takes place if they have been previously mixed with adrenalin and exposed to it for several hours in a slightly alkaline medium. Certain qualitative characteristics of pure adrenalin (Shaw's specificity ratio test) were never observed in the blood results but the qualitative characteristics of the latter correspond closely to those of adrenalin-sterol compounds which had been artificially synthesized in vitro. The process of adrenalin-sterol compound formation is speeded up by alkalinization and reversed by acidification. This also corresponds to Konschegg's findings with his 'lipoid-adrenalin' compounds.

The entire amount of material which appears in the final results obtained with Shaw's modified method in deproteinized blood filtrates can also be recovered in hot, but not in cold alcoholic extracts of the blood. Cholesterol does not participate in the color giving reaction and does not seem to tie up with adrenalin. The latter fact, which has also been stated by Konschegg seems to be true likewise for corticosterone and androsterone. The strongest affinity to adrenalin apparently exists in desoxycorticosterone and in progesterone which is chemically most closely related to desoxycorticosterone and which is also present in the suprarenal cortex.

The quantitative distribution of those substances which contribute to the color reaction in Shaw's method in a given blood specimen cannot be determined directly but the behavior of the specificity ratio (acid:alkaline results) allows a rough estimate of the prevalence of one or the other of them.

In view of the fact that the colorimetric readings of blood filtrates obtained through comparison with a standard adrenalin solution indicate the sum of various substances of different color giving intensities, it is not possible to express the results in terms of weight. It is, therefore, suggested to determine the results in 'color units' based upon the color effect of 1 mγ (billionths of a gram) of adrenalin per cc. With the standard adrenalin solution containing 100 mγ/cc., a blood specimen giving e.g.

an equal color effect would have to be characterized as containing 100 color units per cc., etc.

Despite the interference of ascorbic acid, the modified method of Shaw is useful in the determination of suprarenal secretory activity, both cortical and medullar. In fact, it reveals the presence in the blood of cortico-adrenal compounds which seem to form the bulk of suprarenal internal secretion.

SUMMARY

A modification of Shaw's method for the determination of blood 'adrenalin' is described.

The readings obtained with this modified method are the sums of the colorimetric effects of compounds which consist of sterols of chiefly cortical origin tied up with adrenalin, and of ascorbic acid.

The existence of adreno-cortical ('AC') compounds, their artificial synthesis in vitro and their presence in blood as a natural product of suprarenal cortico-medullar secretion are discussed in detail.

I wish to express my appreciation to Dr. H. B. Pierce, chief of the Biochemistry Department of the University of Vermont for his hospitality and valuable advice; to Dr. E. C. Kendall of the Mayo Clinics for supplying me with corticosterone and compound E; to Ciba Pharmaceutical Products Inc., Summit, N.J. for desoxycorticosterone acetate (percorten), α-estradiol (ovocylin), progesterone (lutocylin), testosterone (perandren), androsterone and Δ-4 androstenedione through the courtesy of Dr. E. Oppenheimer; to the Winthrop Chemical Company, New York (Dr. C. B. Taft) for crystalline vitamin D_2 and to E. R. Squibb & Sons, New York, for crystalline thyroxin.

REFERENCES

1. Shaw, F. H.: *Biochem. J.* 32: 19. 1938.
2. Whitehorn, J. C.: *J. Biol. Chem.* 108: 633. 1935.
3. Bloor, W. R.: *Proc. Am. Soc. Biol. Chemists*, Toronto, April 1939, p. IX.
4. Tietz, E. B., H. Dornheggen and D. Goldman: *Endocrinology* 26: 641. 1940.
5. Hülse, W.: *Ztschr. f. d. ges. exper. Med.* 30: 6. 1920.
6. Stewart, G. N.: *Physiol. Revs.* 4: 163. 1924.
7. Adler, L., *Deutsch. Arch. f. klin. Med.* 114: 283. 1914.
8. Trendelenburg, P.: *Arch. f. exper. Pathol. u. Pharmakol.* 79: 154. 1915.
9. Kure, K., T. Nakaya, S. Murakami and S. Okinaka: *Klin. Wchnschr.* 12: 454. 1933.
10. Konschegg, Th., and J. Monauni: *Ztschr. f. klin. Med.* 131: 99. 1936.
11. Stuber, B., A. Russmann and E. A. Probbsting; *Ztschr. f. d. ges. exper. Med.* 32: 448. 1923.
12. Fraenkel, S., and R. Allers: *Biochem. Ztschr.* 18: 40. 1909.
13. Viale: *Rev. Sud-Amer. de Endocrinol., Immunol. y Quimicoter.* 16: 387. 1915.
14. Crocetta and Dogliotti: *Bull. Soc. Ital. Biol. Sperim.* 8: 443. 1933.
15. Giordano, C., and Zeglio, P.: *Arch per le Scienze Med.* 68: 1. 1939.
16. Stepp, W., J. Kühnau and H. Schröder: Die Vitamine und ihre klinische Anwendung, Ferd. Enke, Stuttgart 1937.
17. Farmer, Ch. J., and A. F. Abt: *Proc. Soc. Exper. Biol. & Med.* 32: 1625. 1935.
18. Stephens, D. J., and E. E. Hawley: *J. Biol. Chem.* 115: 653. 1936.
19. Konschegg, Th.: *Ztschr. f. d. ges. exper. Med.* 81: 559. 1932.
20. Koehler, A. E., and L. Eichelberger: *J. Biol. Chem.* 87: 38. 1930.
21. Kendall, E. C., H. L. Mahon, B. F. McKenzie, C. S. Myers and G. A. Koelsche: *Proc. Staff Meetings Mayo Clinics* 9: 245. 1934.
22. Abelous, Soulié and Toujan: Quoted by Konschegg (19).
23. Kendall, E. C.: *Proc. Am. Soc. Biol. Chem., New Orleans*, March 1940, p. LI.
24. Pfiffner, J. J., and H. B. North: *Am. Soc. Biol. Chem., New Orleans*, March 1940, p. LXXVII.
25. Beall, O., and T. Reichstein: *Nature* 142: 479. 1938.
26. Kutschera-Aichbergen, H. v.: Ueber Herzschwäche, Urban & Schwarzenberg, Berlin–Wien, 1929.
27. Westphal, K., and Ch. Sievert: *Ztschr. f. klin. Med.* 133: 223. 1938.
28. Bennett, H. St.: *Proc. Soc. Exper. Biol. & Med.* 42: 786. 1939.
29. Sjöstrand, T.: *Skandinav. Arch. f. Physiol.* 71: 85. 1935.
30. Rogoff, J. M.: "Das Nebennierenmark" in "Die Drüsen mit innerer Sekretion" Aesculap Verlag Wien-Leipzig, 1937, quoted by Jores (31).
31. Jores, A.: Klinische Endokrinologie, J. Springer, Berlin, 1939.
32. Raab, W.: *Ztschr. f. d. ges. exper. Med.* 105: 657. 1939.

A COMPARISON OF THE BODY AND ENDOCRINE GLAND (ADRENAL, THYROID AND PITUITARY) WEIGHTS OF EMOTIONAL AND NON-EMOTIONAL RATS

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DISSIMILARITY in endocrine gland weights of rats displaying temperamental differences has been reported by several authors. Freudenberger (1) compared the adrenal, thyroid, and pituitary weights of the Wistar with the Long-Evans strain, which he referred to as "nervous," and found heavier adrenals and thyroids but lighter pituitaries in the latter. Donaldson and King (2) and Hatai (3) discovered lighter pituitaries and heavier adrenals in wild Norway rats as compared with Wistar albinos, but practically equal thyroid weights. We have undertaken a similar study of the glands of two strains of rats which have been selectively bred for emotionality and non-emotionality. The work reported here is a comparison of the weights of body and of adrenal, thyroid and pituitary glands of the two psychologically divergent types.

METHOD

The rats used by us are descendants of animals collected from various colonies on the Pacific coast. They were supplied through the courtesy of Dr. Calvin S. Hall, in whose laboratory the strains were developed and who conducted the tests for their emotionality.[1] Additional investigations by Mr. Fred Billingslea (5) of the Psychology Department have demonstrated that the emotional rats are relatively timid and non-aggressive.

All were bred and reared under uniform conditions and fed a diet of Purina Dog Chow and Purina Layena supplemented with milk, yeast, lettuce and cod liver oil. They were given over for our use at various ages. After a 17-hour fast, each rat was killed with ether and weighed. Females were killed during diestrus, as determined by vaginal smears. The adrenals, thyroid and pituitary were removed immediately in the order named, and either weighed at once on an analytical balance, or placed in a glass-stoppered weighing bottle and weighed as soon as the dissection was completed. In either case the same procedure was followed for both the emotional and non-emotional groups. The whole process was carried out as quickly as practicable in order to minimize loss of moisture from the glands. If infection of the lungs or middle ear was discovered upon autopsy, the figures were discarded.

Received for publication August 23, 1940.

[1] Briefly, the test for emotionality is as follows: at 3 months of age, a rat is placed in a large, well lighted, circular enclosure for 2 minutes a day on 12 successive days. This strange situation evokes an emotional response in the rat, including defecation and micturition. A score is obtained by noting the number of days during which the rat either defecates or urinates, while in the enclosure. The emotional strain has been established by interbreeding rats with high scores (many days excretion), the non-emotional strain by interbreeding rats with low scores (no or few days excretion). See Hall (4).

The gland/body ratios were calculated, and the figures grouped according to sex, age and emotionality of the rats. Averages were obtained for each group; and the standard error of each mean, and the standard error of the difference between the emotional and non-emotional means in a group were calculated according to the following formulae.

$$S.E._{mean} = \frac{\sigma}{\sqrt{N}} \qquad S.E._{diff} = \sqrt{(S.E._{mean_1})^2 + (S.E._{mean_2})^2}.$$

The critical ratio was derived by the formula $M_1 - M_2/S.E._{diff}$. If the critical ratio was 3 or more, it was regarded as showing a true difference, occurring by chance only three times in a thousand.

RESULTS

Table 1 presents the mean values of the various absolute weights for each age group, showing the number of emotional and non-emotional rats, the standard error of each mean, and the critical ratio. Adrenal figures represent the average of the two glands; pituitary and thyroid weights are of the entire gland. Generally, the standard error for the emotional rats was found to be larger than that of the non-emotional animals, indicating that the latter strain is perhaps more homogeneous. The difference between body weight averages has no significance in any group, although the females of the two strains display a more marked difference than do the males. Absolute values for the glands show that the emotional males have heavier adrenals and thyroids, but no significant difference exists between the pituitary weights of the two strains. On

TABLE 1. OBSERVED WEIGHTS

	No.	Mean[1] and standard error	Critical ratio	No.	Mean[1] and standard error	Critical ratio
	4-month-old females			*5-month-old males*		
Body	29 E 31 NE	173 ±3.22 164 ±3.25	2.0	37 E 33 NE	254 ±6.01 259 ±7.40	0.5
Adrenals	29 E 31 NE	28.0±0.97 24.9±0.68	2.6	30 E 33 NE	19.1±0.71 16.5±0.43	3.1
Thyroid	29E 31 NE	14.5±0.59 11.5±0.38	4.3	30 E 33 NE	15.5±0.53 12.5±0.42	4.5
Pituitary	26 E 30 NE	10.3±0.27 8.2±0.25	5.7	33 E 29 NE	8.1±0.17 7.8±0.27	0.9
	6 to 7-month-old females			*7 to 8-month-old males*		
Body	31 E 32 NE	198 ±3.88 188 ±3.14	2.0	54 E 60 NE	288 ±5.08 286 ±4.42	0.3
Adrenals	31 E 32 NE	29.8±0.65 25.9±0.70	4.1	54 E 60 NE	21.8±0.58 18.8±0.38	4.3
Thyroid	31 E 32 NE	14.7±0.78 12.0±0.62	2.7	53 E 60 NE	18.5±0.58 14.4±0.38	5.9
Pituitary	22 E 24 NE	11.4±0.47 8.4±0.43	4.7	45 E 53 NE	8.6±0.16 8.1±0.17	2.2

E=emotional rats, NE=non-emotional rats.
[1] Body weight in grams, other weights in milligrams.

the other hand, the female rats of both age groups show a consistently significant difference in respect to the pituitary weights only. The adrenal weights of the 4-month females and the thyroid weights of the older females reveal merely a tendency toward greater weight in the emotional animals; none of the critical ratios for these glands is as convincing as those of the pituitaries.

The ratios of gland weight to body weight are presented in table 2. On the whole, the differences between the strains, as seen by the critical ratios, are accentuated in the older rats. In the males of both age groups the same relationships hold as were found for the observed gland weights, namely, the adrenal/body and thyroid/body

TABLE 2. RELATIVE WEIGHTS

	No.	Mean[1] and standard error	Critical ratio	No.	Mean[1] and standard error	Critical ratio
	4-month-old females			*5-month-old males*		
Adrenals/ Body	29 E 31 NE	0.161±0.0045 0.153±0.0051	1.2	30 E 33 NE	0.073±0.0025 0.064±0.0014	3.4
Thyroid/ Body	29 E 31 NE	0.083±0.0031 0.071±0.0025	3.0	30 E 33 NE	0.059±0.0020 0.049±0.0019	3.6
Pituitary/ Body	26 E 30 NE	0.058±0.0011 0.046±0.0011	5.8	33 E 29 NE	0.030±0.0008 0.029±0.0006	1.0
	6 to 7-month-old females			*7 to 8-month-old males*		
Adrenals/ Body	31 E 32 NE	0.151±0.0036 0.138±0.0032	2.7	54 E 60 NE	0.076±0.0016 0.066±0.0011	5.2
Thyroid/ Body	31 E 32 NE	0.074±0.0022 0.064±0.0018	3.6	53 E 60 NE	0.064±0.0018 0.051±0.0012	6.0
Pituitary/ Body	22 E 24 NE	0.058±0.0018 0.046±0.0021	4.7	45 E 53 NE	0.031±0.0007 0.029±0.0005	2.3

E=emotional rats, NE=non-emotional rats.
Body weight in grams, other weights in milligrams.

weights differ significantly, but the pituitary/body weights do not. In the female groups, on the other hand, the pituitary ratios show the most significant differences; the difference between the thyroid/body weights is very likely a true one, but the adrenal figures are almost as non-significant as the figures for the male pituitaries.

DISCUSSION

The rats of this investigation evidently do not conform to the types compared by Donaldson, Hatai and Freudenberger. The Long-Evans strain (1) is characterized by heavier adrenals and thyroids, but lighter pituitaries than the Wistar rats. The wild Norway rats also possessed heavier adrenals and lighter pituitaries than the Wistar strain, but did not differ from it in thyroid weight (2, 3). The emotional animals of the present study have heavier pituitaries in the females and apparently the same weights in the males, as well as heavier thyroids for both sexes.

The non-significant difference between pituitary weights of emotional and non-emotional males seems surprising, for two reasons: first, a paper by Anderson and Anderson (6), dealing with the gland weights of emotional and non-emotional male rats chosen from an apparently homogeneous colony, states that the pituitaries were

the only endocrines of the three investigated (the others being the adrenals and thyroid) which showed a significant difference between the psychological types. These results are directly opposed to ours.

Secondly, the female pituitary weights reveal an unmistakably significant difference. The discrepancy between the sexes can scarcely be due to faulty technic, since removal of the pituitary involves no judgment as to the extent of margins, as with the thyroid, and no dissection of possibly unequal amounts of connective tissue, as with the adrenals. There is more reason to expect reliability in these gland weights than in any other. The only known difference between the treatment of the sexes was the killing of the females at diestrus. The possibility that the male pituitary goes through a cyclic weight change suggests itself.

It is impossible to claim any cause-and-effect relationship between the gland weight differences and the emotional reactions of the animals. However, if there be any relation at all, it is obvious that the thyroids alone, of the three endocrines investigated, show a significant difference in weight between the emotional and non-emotional animals of both sexes. The work of Anderson and Kennedy (7) on female rat adrenals demonstrated their great fluctuations in weight during the estrous cycle. Although every precaution was taken by us to kill the females during diestrus, failure to do so at precisely identical periods in the cycle may account entirely or in part for the lack of significant difference between the two strains. But judged on the evidence at hand, the adrenals in the female and the pituitaries in the male show no correlation with emotionality.

In regard to the thyroid weights, we cannot ignore the possibility that the emotional strain exhibits a tendency toward hypertrophy of that gland, which may have nothing to do with the psychological reactions. However, preliminary investigations by one of us on rats that have been made "neurotic" by the air-blast technic (8) show a tendency in these animals toward enlarged thyroids. If subsequent investigation supports these findings, it would indicate that the increased thyroid weight is a result, not a cause, of the abnormal reaction of the animal, as is true in many clinical cases of thyroid hypertophy; and perhaps the same conclusion may be drawn for the emotional and non-emotional animals reported here.

SUMMARY

Emotional and non-emotional rats of comparable age and sex do not differ significantly in body weight.

Male emotional rats have heavier adrenals and thyroids, but their pituitary weights do not differ significantly from those of the non-emotional males.

The thyroid, and especially the pituitary weights of emotional females are significantly different from those of non-emotional females. The adrenal weight differences are probably non-significant.

The thyroid gland alone in both sexes is consistently heavier in the emotional animals.

REFERENCES

1. Freudenberger, C. B.: *Am. J. Anat.* 50: 293. 1932.
2. Donaldson, H. H., and H. D. King: *Am. Anat. Mem.* No. 14. Philadelphia. 1929.
3. Hatai, S.: *Anat. Rec.* 8: 511. 1914.
4. Hall, C. S.: *J. Comp. Psychol.* 18: 385. 1934.
5. Billingslea, F.: *J. Comp. Psychol.* (in press).
6. Anderson, E. E., and S. F. Anderson: *J. Comp. Psychol.* 26: 459. 1938.
7. Anderson, D. H., and H. S. Kennedy: *J. Physiol.* 76: 247. 1932.
8. Hall, C. S., and R. F. Martin: *J. Psychol.* 10: 207. 1940.

EFFECT OF CASTRATION IN THE DILUTE BROWN STRAIN OF MICE[1]

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IN CONNECTION WITH A STUDY of the effect of early ovariectomy upon the Jackson Laboratory dilute brown strain of mice, nodular hyperplasia of the adrenal cortex similar to that which occurred in the females (1) was noted in a few castrate male mice. An attempt was made to determine the regularity and behavior of the hyperplasia in the male and also to see if there was any indication of sexual stimulation when this was present. The adrenals, the accessory sex glands and in some cases the pituitary, thyroid, parathyroid and thymus were saved for study in serial section. The skins were fixed and the growth of mammary rudiments observed. Some of the mammary glands were studied histologically. Forty males were castrated at birth and 60 at later ages. This is a report on those which have died or have been killed up to the present time. Mice excluded are those which were too decomposed for study or those dying from definite diseases. An equal number of unoperated males were set aside and these with additional stock males have served as controls.

In the unoperated males the adrenal cortex was normal. The accessory sex glands were well developed and normal. In 11 males examined beyond 550 days of age (average 642.3 days) we found the mammary duct development to be only rudimentary. Some of the mammary rudiments could not be found and were possibly absent. The longest was about 0.3 cm.

Thirteen mice which were castrated at one day of age were observed when beyond 10 months. All have shown nodular hyperplasia in at least one adrenal and to a degree where it could be detected without, although confirmed by, histological examination. In most cases the left adrenal exhibited the abnormality to a greater extent than the right adrenal. The degree of hyperplasia increased markedly with the age of the animal, but in no case has it become as extreme as with some of the ovariectomized mice reported on (fig. 1–2, 4–5). In both male and female the early stages always seem to consist of non-pigmented cells. The yellow-brown pigment which is very evident with the later ages of the female has not been prominent in the adrenal of the male. In general one gains the impression that the whole hyperplastic development is less advanced at any one given age in the male than in the female.

In all castrated mice the accessory sex glands were regressed.

Two mice examined at 10, and 4 examined at 13 months of age failed to show any unusual development of the mammary ducts. However, one killed at 437 days of age showed marked growth in 3 of the 10 glands. Six mice reaching more than 500 days of age (average 644.2 days) have shown extensive mammary gland growth (fig. 3). All 10 glands have not grown in any one of these mice but all pairs of glands are represented within the group. In the 6 animals there was a possibility of 60 glands, but as

Received for publication September 5, 1940.
[1] Supported in part by a grant from the Josiah Macy, Jr. Foundation.

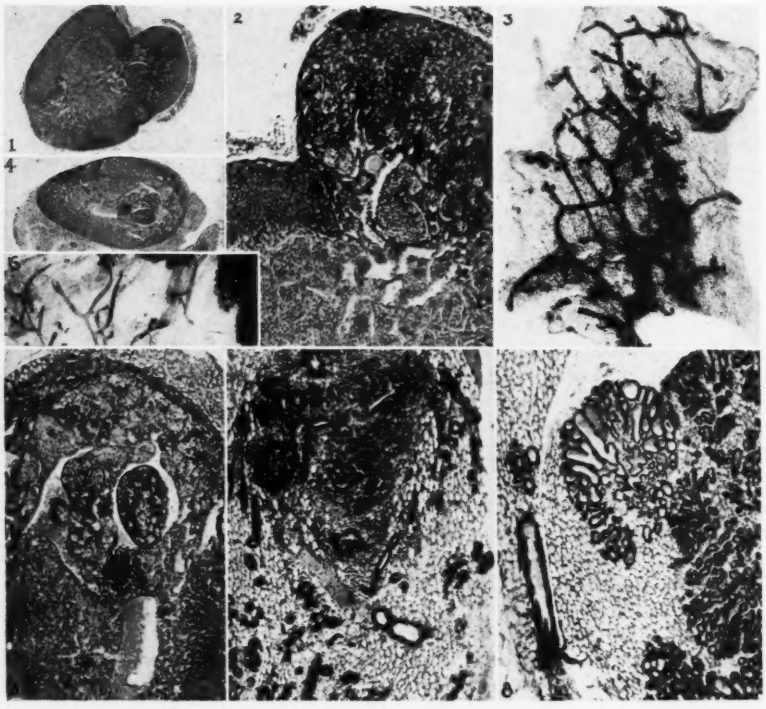

Fig. 1. Adrenal of male *mouse P1401* castrated at birth and killed at 589 days of age showing nodular hyperplasia of the adrenal cortex. Hematoxylineosin (used for all histological illustrations), ×13. Fig. 2. Part of adrenal cortex of male *mouse P1401* showing hyperplastic area, ×44. Fig. 3. Whole mount of mammary gland of male *mouse P1401* castrated at birth and killed at 589 days of age, ×7. Fig. 4. Adrenal of male *mouse P1446* castrated at birth and killed at 645 days of age showing nodular hyperplasia of the adrenal cortex. Fig. 5. Part of adrenal cortex of male *mouse P1446* showing hyperplastic area, ×44. Fig. 6. Whole mount of mammary gland of male *mouse P1446* showing two different types of gland growth within the same animal, ×5. Fig. 7. Third mammary gland of male *mouse P1446* showing malignant changes, ×44. Fig. 8. Third mammary gland of male *mouse P1446* showing adenomatous hyperplasia, ×44.

is shown in the table, only 28 developed. There has been a great variation in the

Pair number (anterior to posterior)	1	2	3	4	5
Number of glands developing	7	4	4	11	2

extent of development of the various glands in the same animal. In one instance the duct system extended over an area 2 cm. in length. The type of development as determined by sub-branching and presence of alveoli has been more uniform within the glands of each male than between the males. One male killed at 589 days of age had 2 tumorous growths, one in each of the third pair of glands. On the left side the third gland consisted mostly of simple duct growth with no great overlapping. The ducts extended finger-like over an area 1.5 cm. in length. In one small area the ducts were several layers thick, forming a dense mass having somewhat the appearance of the gland of a 15-day pregnant female (fig. 6). This region contained a mammary carcinoma (fig. 7). A similar but less extensive area of overgrowth appeared in the third gland of the right side. This contained adenomatous hyperplasia of the mammary gland (fig. 8).

An enlargement of the pituitary gland could easily be seen before it was sectioned. Upon histological examination it was found that the anterior lobe was enlarged and showed marked congestion. Castration cells were not present.

Although the first series of castrations were made at one day of age, this age is not an essential feature in securing adrenal and mammary gland changes. (This is also true for the female because we have found that ovariectomy at 15 months of age has been followed by development of nodular hyperplasia of the adrenal.) In a male castrated at 30 days of age and killed at 582 days both adrenals were abnormal and the mammary ducts had shown extensive growth. A male castrated at 6 months and killed 3 months later had beginning hyperplasia of the adrenals but no mammary duct growth. Another male castrated at 6 months and killed 9 months later had extensive adrenal changes and good duct growth.

DISCUSSION

Castration as well as ovariectomy has brought out interesting characteristics in the dilute brown strain of mice. With this method of attack the adrenal gland may well become a sensitive scale upon which heretofore obscure physiological activities may be measured. Work is partially complete which attempts to compare the results of gonadectomy within a number of strains of mice. Fortunately inbreeding had already prepared a number of strains upon which studies could be made.

The results within the dilute browns show that the removal of either the male or the female gonads is followed by development of hyperplastic adrenal cortex changes. (This should not be confused with brown degeneration of the adrenals. The early stages and development of nodular hyperplasia have been traced in the female and will be presented in a later publication.) The presence of even one ovary is sufficient prevent the hyperplastic changes in the cortex. With the removal of the gonads in either the male or the female changes characteristic of feminization appear. Attention has previously been called to the stimulation of uterus, vagina and mammary glands after ovariectomy. That the stimulation is female-like rather than male-like after castration is evidenced by the lack of development of the accessory sex glands and the growth of the mammary glands. The fact that the castration cells are absent from the pituitary is suggestive that the effect is not due entirely to this gland. The proportion of the different cell types in the pituitary needs further study than has been possible up to the present time. Although there is no direct evidence that the hyperplastic adrenal cortex is the source of the feminizing influence, it is interesting that there has always been an adrenal change where this has occurred. Furthermore, the hyperplastic changes have preceded the feminization. In later ages of the animal the degree of feminization has been directly related to the degree of cortical hyperplasia.

Another point of interest is that preliminary evidence has indicated that marked nodular hyperplasia of the adrenal cortex appears only in the later periods of the life of the animal. The changes also appear more rapidly after castration in later life. More evidence on these matters is being secured.

SUMMARY

In male mice of the dilute brown strain, castrated at birth, or at one month or at 6 months of age, hyperplasia of the adrenal cortex and in many cases growth of the mammary glands has been observed. The changes of the adrenal preceded the mammary gland development. In one of the males which was castrated at birth, two mammary tumors have appeared.

REFERENCE

1. Woolley, G., E. Fekete and C. C. Little: *Proc. Nat. Acad. Sci.* 25: 277. 1939.

NOTES AND COMMENTS

ESTROGENS IN THE URINE OF NORMAL AND VITAMIN E DEPLETED RATS*

D'AMOUR, FUNK AND GLENDENNING[1] claim that normal female rat urine contains about three rat units per liter of estrogen, and that this increases over a straight line curve to ten rat units per liter in the third week of pregnancy. Gahrbandt[2] and Szorka and Kuntz[3] claim that vaginal estrus is produced in the rat by about 0.8 gamma of ketohydroxyestrin, and uterine estrus by 4.8 gamma.

Control experiments showed that the average daily urine secretion per rat in our laboratory was about 5 cc. Urine was collected by absorbtion on towels, placed beneath the screen of false bottom nutrition cages. The urine was calculated in rat days and then converted to liters to facilitate comparison. One rat day equals 0.005 liter. The method of extraction and assay used was the same as that employed by D'Amour, Funk and Glendenning.[1] Pregnant rats were put in cages, according to the stage of pregnancy, four arbitrary stages being selected. Normal females of various ages were also put in different cages. Large amounts of urine were collected over long periods from normal females placed in cages daily that corresponded with the types of vaginal smear i.e. estrual cycle.[4] Urine was also collected from normal males. Having at our disposal a large number of vitamin E depleted female rats, data was collected on their pregnancy urine.[5] These results are all summarized in table 1.

TABLE 1

Type of rat	R.U./liter urine	Type of rat	R.U./liter urine
Normal male	none in 5 liters	Normal pregnant	
Normal female		female 1–5 days	4.5
Age 25– 75 days	2.5	5–10 days	7.5
75–150 days	4.0	10–15 days	12.0
150–250 days	4.0	15–18 days	15.0
Vitamin E depleted		18–21 days	17.0
female	3.5	Vitamin E depleted	
Females in estrual		pregnant females	
stages a.	3.0	1– 5 days	4.5
b.	3.0	5–10 days	7.5
c.	3.5	10–15 days	11.0
d.	5.0	15–18 days	13.5
e.	3.5	18–21 days	13.0

Summary. The results indicate that female rat urine shows a regular increase in estrogen of about 66% once in 5 days, corresponding approximately with a proestrous vaginal smear. Increase in urine estrogen during pregnancy is about 6 times the normal value. Pregnant vitamin E depleted females showed normal pregnancy values for about 12 days, giving slightly low values until about 18 days, when the estrogen fell off abruptly. This drop approximated the drop in weight of the resorbing fetuses. All values were roughly characteristic for mammals. Thirty liters of rat urine was collected to obtain these figures.

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Received for publication October 1, 1940.

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[1] D'AMOUR, F. E., D. FUNK AND M. D. GLENDENNING: *Proc. Soc. Exper. Biol. & Med.* 35: 26. 1936.
[2] GOHRBANDT, E.: *Klin. Wchnschr.* 16: 681. 1937.
[3] SZARKA, S., AND G. KURTZ: *Endocrinology* 23: 64. 1938.
[4] ALLEN E., AND E. A. DOISY: *Am. J. Anat.* 34: 169. 1924.
[5] EVANS, H. M., AND G. O. BURR: *University of California Memoirs* 8: 1. 1927.